# Biosynthesis of plant polyketides in yeast

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Dissertation von

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# Table of Content

# 1 Summary

Industrial biotechnology aims to replace production processes based on petrochemicals with more sustainable biological processes based on renewable raw materials. With the rise of metabolic engineering and synthetic biology in the last decades, the range of products attainable by this technology has widened substantially. This thesis explores the potential of *Saccharomyces cerevisiae* for the production of two commercially interesting compound classes within the plant polyphenols.

The first part demonstrates heterologous production of various dihydrochalcones. A side activity of the native ScTsc13, the reduction of *p*-coumaroyl-CoA to *p*-dihydrocoumaroyl-CoA, was used for *de novo* production of phloretin, the first committed dihydrochalcone. By further extension of the pathway from phloretin, by employing decorating enzymes with known specificities for dihydrochalcones, and by exploiting substrate flexibility of enzymes involved in flavonoid biosynthesis, *de novo* production of the antioxidant molecule nothofagin, the antidiabetic molecule phlorizin, the sweet molecule naringin dihydrochalcone, and 3-hydroxyphloretin was achieved.

In the second part, yeast was engineered for *de novo* production of anthocyanins, molecules that are used in the food and beverage industries as natural colorants. Enzymes from different plant sources were screened and efficient variants were found for most steps of the pathways to the three main anthocyanins. However, as previously shown *in vitro* and in *Escherichia coli*, the shunt flavonol production by the anthocyanidin synthase was a major limitation.

In the third part, this flavonol by-product formation was eliminated by co-expression of glutathione-S-transferases. These enzymes, previously thought to be involved in vacuolar transport of anthocyanins in plants, were shown to be required for correct product formation by anthocyanidin synthases. By additional co-expression of glycosyltransferases and a malonyltransferase, the pathway was extended to various decorated anthocyanins with a range of different colors.

This thesis uncovers and describes important steps towards a sustainable biotechnological process for production of dihydrochalcones and anthocyanins. However, further optimization to increase titers will be required before such processes become commercially viable.

# 2 Zusammenfassung

Die industrielle Biotechnologie ermöglicht es, petrochemische Produktionsprozesse durch nachhaltigere biologische Prozesse auf Basis von nachwachsenden Rohstoffen zu ersetzen. In den letzten Jahrzehnten hat sich mit dem Aufkommen von Metabolic Engineering und der synthetischen Biologie das Produktspektrum der industriellen Biotechnologie erheblich erweitert. Diese Dissertation untersucht das Potenzial von *S. cerevisiae* zur Herstellung von zwei kommerziell interessanten Stoffklassen, die zu den pflanzlichen Polyphenolen gehören.

Der erste Teil zeigt die heterologe Produktion von verschiedenen Dihydrochalconen. Zur *de novo* Produktion von Phloretin, dem Ausgangsmolekül der meisten Dihydrochalconen, wurde die Reduktion von *p*-cumaryl-CoA zu *p*-dihydrocumaryl-CoA durch ScTsc13 verwendet, eine Nebenaktivität dieser endogenen Doppelbindungsreduktase. Der Stoffwechselweg wurde dann unter Verwendung von Enzymen mit bekannten Spezifitäten und durch Ausnutzung der Substratflexibilität von Enzymen, die an der Flavanoidbiosynthese beteiligt sind, zum antioxidativen Molekül Nothofagin, zum antidiabetischen Molekül Phloridzin, zum Süßstoff Naringin Dihydrochalcon und zu 3-Hydroxyphloretin erweitert.

Im zweiten Teil wurde gezeigt, dass Hefe zur *de novo* Produktion der drei wichtigsten Anthocyanen, welche in der Lebensmittel- und Getränkeherstellung Anwendungen als natürliche Farbstoffe haben, verwendet werden kann. Enzyme aus verschiedenen Pflanzen wurden getestet und effiziente Varianten für die meisten Schritte des Stoffwechselwegs wurden gefunden. Wie zuvor schon für *in vitro* Reaktionen und in *E. coli* gezeigt wurde, war die Akkumulation der Flavonol Nebenprodukte aufgrund der Anthocyanidinsynthase auch in Hefe das Hauptproblem.

Im dritten Teil wurde die Bildung der Flavonol Nebenprodukte durch die Koexpression von Glutathion-S-Transferasen eliminiert. Für diese Enzyme wurde vorher angenommen, dass sie am vakuolären Transport von Anthocyanen in Pflanzen beteiligt sind. Sie werden jedoch für die korrekte Produktbildung der Anthocyanidinsynthase benötigt. Durch zusätzliche Expression von Glykosyltransferasen und einer Malonyltransferase konnten wir die *de novo* Produktion von verschieden dekorierten Anthocyaninen mit unterschiedlichen Farben zeigen.

Diese Dissertation ist ein wichtiger Schritt zu einer nachhaltigeren biotechnologischen Produktion von Dihydrochalconen und Anthocyaninen in Hefe. Um

die notwendigen Produktionstiter für einen kommerziell umsetzbaren Prozess zu erreichen sind jedoch noch weitere Optimierungen erforderlich.

# 3 Introduction

## 3.1 Industrial biotechnology in the age of synthetic biology

### 3.1.1 Brief history of industrial biotechnology

Microorganisms have been used for preservation and enhancement of foods and beverages long before their exact nature was discovered. Earliest records of the use of fermentation date back to 7000BC, when in Sumeria and Babylonia the conversion of sugar to alcohol was used to brew beer (Demain, 2010). Ever since, all around the world, a plethora of fermented foods have been produced and consumed (Katz, 2012). The use of large-scale microbial fermentation for non-food products was pioneered during World War I, when in Germany yeast was used for conversion of sugars into glycerol and in the United Kingdom *Clostridium* was shown to produce acetone and butanol (Demain, Vandamme, Collins, & Buchholz, 2016).

In the following decades of the 20$^{th}$ century, industrial biotechnology mainly focused on primary and secondary metabolites produced natively in various microorganisms, such as antibiotics, amino acids, nucleotides, vitamins, organic acids, or alcohols. While initial strain improvement was based on cycles of random mutagenesis and screening, selection strategies were introduced in the 1950s, in order to reduce the numbers of screened strains (Demain, 2010). In the late 1970s and 1980s, major breakthroughs in recombinant DNA technology enabled new approaches for production of chemicals, but also biotechnological production of protein biopharmaceuticals, such as human insulin, or of industrial enzymes, e.g. for food processing and as detergents (Demain, 2010; Headon & Walsh, 1994). Further development of analytical technologies, and the emphasis on the importance of metabolic fluxes led to the rise of a novel concept called metabolic engineering in the 1990s. In the seminal book of the field it was defined as “the directed improvement of product formation or cellular properties through the modification of specific biochemical reaction(s) or the introduction of new one(s) with the use of recombinant DNA technology“ (Stephanopoulos, Aristidou, & Nielsen, 1998).

Driver of the next revolution in industrial biotechnology was the standardization and characterization of well-defined parts (e.g. enzymes, expression units, localization tags, integration sites), novel DNA assembly and genome scale engineering

technologies, as well as the emergence of cost-effective DNA sequencing and synthesis, which led to the field of synthetic biology (Wittmann, 2017). Synthetic biology is based on an engineering approach (design-build-test-learn cycle) to rationally design organisms or other biological systems not found in nature (König, Frank, Heil, & Coenen, 2013). This has resulted in a huge expansion of the product range of industrial biotechnology to various plant natural products such as steviol glycoside sweeteners (WO/2011/153378, 2011), opioid painkillers (Galanie, Thodey, Trenchard, Interrante, & Smolke, 2015), or the antimalarial drug precursor artemisinic acid (Paddon & Keasling, 2014), but also to platform chemicals like butanediol (Burgard, Burk, Osterhout, Van Dien, & Yim, 2016), propanediol (Nakamura & Whited, 2003), or farnesene (Meadows et al., 2016).

Besides impacting the chemical industry, industrial biotechnology is becoming more and more visible in mainstream consumer products. Adidas, Patagonia, and North Face have all rolled out fermentation based spider silk clothing prototypes soon to be mass produced (Scott, 2017). Lego and Coca-Cola are both aiming to replace their fossil-based plastics with biotechnology derived sustainable alternatives (Lego, n.d.; Virent, n.d.). Even supermarket shelves might soon be reached by industrial biotechnology, with Perfect Day Foods aiming to launch milk produced by engineered yeast in 2018 (Perfect Day, n.d.). With biotechnology now impacting many aspects of human life, the term bioeconomy was born, which the Organization for Economic Co-operation and Development (OECD) defines as the share of the economy delivered by biotechnology (Flores Bueso & Tangney, 2017).

### 3.1.2 Impact of industrial biotechnology

In 2012, the US biotechnology sector had estimated total revenues of $324 billion, which amounted to over 2% of the gross domestic product. These revenues were further split between three sectors: biologics (drugs) at $91 billion, crops at $128 billion, and industrial products (biofuels, enzymes, biomaterials, and biochemicals) at $105 billion. Over the past decade, the US biotechnology industry has grown with annual growth rates >10%, which was far above the total economy (Carlson, 2016). Deloitte, a financial consulting business, valued the global fermentation based industry at $127 billion in 2013. Ethanol accounted for 94% of the total production volume, while generating 87% of the value. The four next biggest products were lysine, glutamic acid, citric acid, and lactic acid, which accounted for 89% of the higher margin products

(Deloitte, 2014). Synthetic biology applications in industrial biotechnology were projected to grow even more, and their global value of $3.9 billion in 2016 is estimated to reach $11.4 billion by 2021 (Flores Bueso & Tangney, 2017).

Economic growth has been linked to negative environmental impact ever since the industrial revolution. However, industrial biotechnology allows for growth, while at the same time saving water, energy, raw materials, and waste production. It is estimated that the field was already saving emissions of 33 million tons of $CO_2$ in 2011, with a potential of 1 to 2.5 billion tons by 2030. This would exceed the total reported emissions of Germany in 1990 (OECD, 2011). Table 1 summarizes life cycle analyses of several industrial biotechnology products on market, showing the enormous potential impact on greenhouse gas emission and energy savings compared to petroleum based production.

**Table 1.** Savings of greenhouse gas emission (GHGE) and energy usage for production by fermentation versus petrochemical production of various products on market.

| Product | Company | GHGE savings | Energy savings | Reference |
|---|---|---|---|---|
| Succinic acid | BioAmber | 100% | 61% | (BioAmber, 2013) |
| 1-3-propanediol | DuPont Tate & Lyle | 56% | 42% | (DuPont Tate & Lyle, n.d.) |
| Riboflavin | Hoffmann – La Roche | 100% | 0% | (OECD, 2001) |
| Polylactic acid polymer | NatureWorks | 30-60% | 30-50% | (Vink, Davies, & Kolstad, 2010) |

Current industrial biotechnology processes mainly use sugars derived from corn, wheat, and sugarcane as raw materials, which are also used as human and animal feedstock. This lead to the so-called food versus biofuel debate (Ajanovic, 2011). A link between rising food prices and decreasing food availability with increasing biofuel production remains questioned (Mohr & Raman, 2013). Recent developments should however resolve this debate. Several full-scale second generation bioethanol plants are now in use, which are able to run on lignocellulosic waste streams from agriculture, such as corn stover or straw, as feedstocks (Jansen et al., 2017).

### 3.1.3 Industry landscape and recent trends

Many large chemical and food companies like Ajinomoto, Archer Daniels Midland, BASF, Cargill, DowDuPont, Evonik Industries, Kyowa Hakko Kirin, Novozymes, or Royal DSM have a long history of using industrial biotechnology for

production of amino acids, organic acids, biopolymers, vitamins, lipids, or enzymes (Dechema, 2004; Research and Markets, n.d.). With the rise of metabolic engineering in the 1990s and synthetic biology in the 2000s, many new industrial biotechnology companies have been established (Tables 2,3).

**Table 2.** List of public industrial biotechnology companies. Financial results are extracted from annual reports 2016. IPO: initial public offering.

| Name | Location | Founded | IPO | Field | Net income/(loss) |
|---|---|---|---|---|---|
| Aemetis | US | 2005 | 2007 | Biofuels | (15'636'000) |
| Amyris | US | 2003 | 2010 | Specialty chemicals | (87'334'000) |
| BioAmber | US | 2008 | 2013 | Specialty chemicals | (28'371'000) |
| Brain AG | DE | 1993 | 2016 | Enzymes/Organisms | (8'700'000) |
| Codexis | US | 2002 | 2010 | Protein Engineering | (8'558'000) |
| Deinove | FR | 2006 | 2010 | Antibiotics/Nutrition | (6'279'000) |
| Evolva | CH | 2004 | 2009 | Health & nutrition | (35'800'000) |
| Gevo | US | 2005 | 2011 | Biofuels | (37'228'000) |
| Metabolic Explorer | FR | 1999 | 2007 | Specialty chemicals | 6'623'000 |
| TerraVia | US | 2003 | 2011 | Health & nutrition | (101'556'000) |

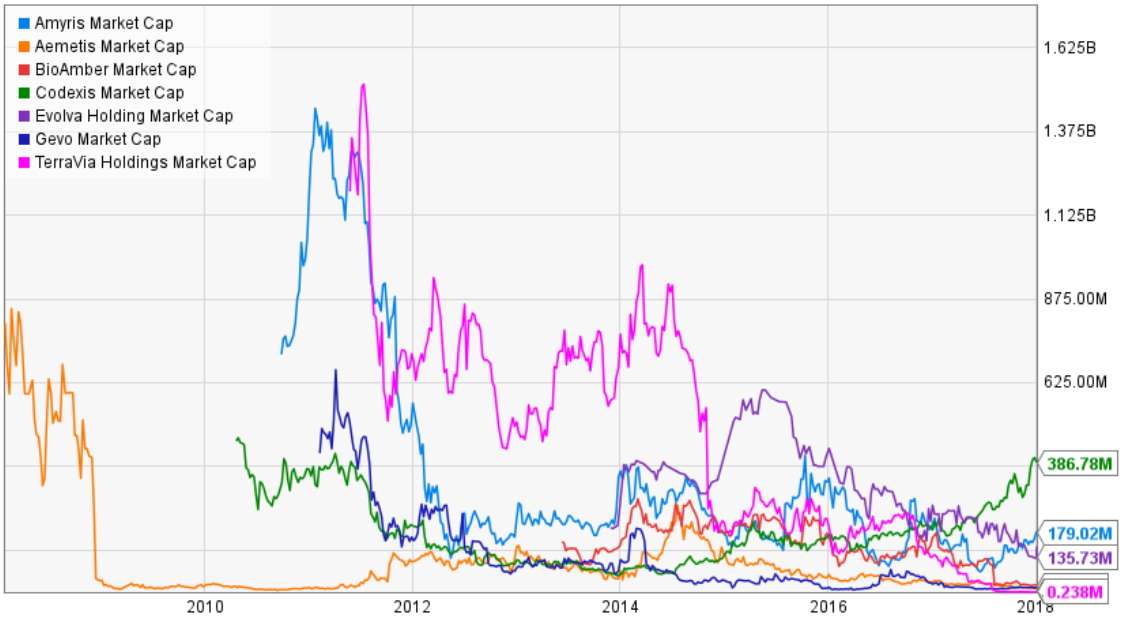


**Figure 1.** Market capitalization (in US$) of public industrial biotechnology companies. Generated at (YCharts, n.d.).

Around 2010, there was a big hype about the first wave of successful companies and several of them became publicly traded (Table 2). Many of them already had first products on the market or were shortly before introducing them. Figure 1 shows the development of the market capitalization of these publicly traded companies. After this initial hype, there was a large drop in valuation for most of them. They were confronted with various problems, ranging from problems in scale-up and large scale production,

delayed launch of key products, higher than expected production costs to slower than promised market penetration of launched products (Cumbers, 2014; Lane, 2016, 2017a, 2017b; Lievense, 2016). Except for Metabolic Explorer, all these public industrial biotechnology companies were still making losses in 2016 (Table 2).

Besides the publicly traded companies, a vibrant ecosystem of well-funded, private industrial biotechnology startup companies has developed (Table 3). In recent years, funding in synthetic biology companies has been growing heavily (Figure 2), and Ginkgo Bioworks was recently declared a "unicorn", a privately held company valued at over $1 billion (Lee, 2017).

**Table 3.** List of well-funded private industrial biotechnology companies. Total funding raised by companies extracted from (Crunchbase, n.d.).

| Name | Location | Founded | Funding (US$) | Field |
|---|---|---|---|---|
| Anellotech | US | 2008 | 27'250'000 | Specialty chemicals |
| AgriMetis | US | 2014 | 30'800'000 | Natural crop protection agents |
| Algenol | US | 2006 | 25'000'000 | Algae biofuels |
| Arzeda | US | 2008 | 15'200'000 | Protein design |
| Asilomar Bio | US | 2012 | 15'250'000 | Natural crop protection agents |
| Bolt Threads | US | 2009 | 212'999'999 | Spider silk |
| Calysta Energy | US | 2011 | 88'000'000 | Gas fermentation |
| DNA Script | FR | 2014 | 13'500'000 | DNA synthesis |
| Ecovative | US | 2007 | 20'054'293 | Performance materials |
| Genomatica | US | 2000 | 137'124'621 | Strain & process engineering |
| Ginkgo Bioworks | US | 2008 | 429'120'000 | Organism design |
| Green Biologics | GB | 2003 | 120'110'515 | 2nd generation feedstocks |
| Greenlight Biosciences | US | 2008 | 46'000'000 | Cell free production |
| Inscripta | US | 2015 | 23'000'000 | Genome editing |
| LanzaTech | US | 2005 | 204'300'000 | Gas fermentation |
| LifeMine Therapeutics | US | 2016 | 55'000'000 | Eukaryotic microbial drugs |
| Lumen Bioscience | US | 2017 | 13'000'000 | Spirulina platform |
| Lygos | US | 2010 | 13'120'000 | Specialty chemicals |
| Modern Meadow | US | 2011 | 53'500'000 | Cultured leather |
| Myriant | US | 2005 | 110'000'000 | Specialty chemicals |
| Provivi | US | 2013 | 29'471'105 | Natural crop protection agents |
| Synthace | GB | 2011 | 16'277'314 | SynBio operating system |
| Teewinot Life Sciences | US | 2015 | 19'300'000 | Cannabinoids |
| Transcriptic | US | 2012 | 27'770'000 | Automation/Cloud lab |
| Sphere Fluidics | GB | 2010 | 15'004'627 | Single cell analysis |
| Spiber | JP | 2007 | 148'090'000 | Spider silk |
| Sweetwater Energy | US | 2006 | 28'591'612 | 2nd generation feedstocks |
| Synthetic Genomics | US | 2005 | 40'000'001 | Organism design |
| Twist Bioscience | US | 2013 | 203'110'714 | DNA synthesis |
| Verdezyne | US | 2005 | 86'466'000 | High value chemicals |
| Zymergen | US | 2013 | 174'140'000 | Machine learning/automation |

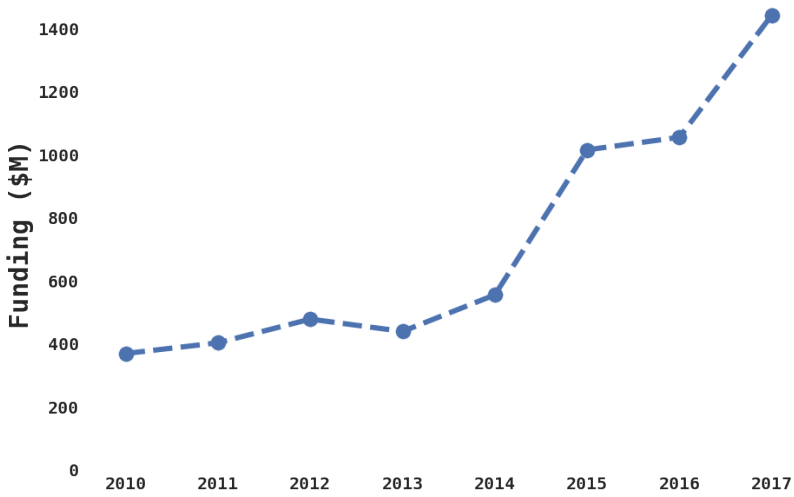


**Figure 2.** Development of total venture funding raised by synthetic biology companies. From (Schmidt, n.d.).

These private companies are a good indicator of the recent trends in industrial biotechnology and synthetic biology. Forty years after Sanger sequencing was developed, sequencing costs are continually dropping and read lengths are increasing, driven by companies like Illumina, Pacific Biosciences, or Oxford Nanopore Technologies (Shendure et al., 2017). Besides increasing the amount of sequenced genes and organisms, this allows for novel experimental approaches. Deep sequencing can be used as readout of pooled growth coupled assays (A. M. Smith et al., 2009), flow cytometry experiments (Bonde et al., 2016), or adaptive lab evolution (Lang et al., 2013). Similarly, startup companies like Twist and Gen9 (acquired by Ginkgo Bioworks in 2017 (Ginkgo Bioworks, n.d.-b)) are disrupting the DNA synthesis market and prices have dropped from 1$ to a few cents per base pair in the last decade (Dance, 2016). Therefore, screening thousands of synthesized enzyme variants is now feasible (Ginkgo Bioworks, n.d.-a), and synthesis of the SC2.0 *Saccharomyces cerevisiae* genome as the first synthetic eukaryotic genome is well underway (Kannan & Gibson, 2017). In order to unleash the full potential of these developments and by taking advantage of novel DNA assembly technologies (Casini, Storch, Baldwin, & Ellis, 2015), companies like Amyris, Ginkgo Bioworks, or Zymergen have created fully automated workflows of DNA assembly, strain construction, and strain characterization. Together with big data and machine learning approaches for understanding and interpreting the results, this allows for a much larger scale of experiments (Ginkgo Bioworks, n.d.-c; Platt, 2015; Zymergen, n.d.). Systems biology technologies also have a large impact on industrial biotechnology applications. As an example, Genomatica has built an integrated

biotechnology platform, incorporating genomics, transcriptomics, proteomics, metabolomics, and fluxomics with genome scale metabolic models for validation and optimization of strains (Barton et al., 2014). Advances in computational methodologies and computational power have also enabled the field of *de novo* protein design, a technology now being commercialized by Arzeda (Arzeda, n.d.; P.-S. Huang, Boyken, & Baker, 2016). Finally, genome engineering was revolutionized in the last couple of years with the development of MAGE and CRISPR-Cas9. These tools allow multiplexing of genome engineering steps, leading to shorter design-build-test-learn cycles (Bao, Cobb, & Zhao, 2016), as well as making less studied organisms of industrial interest genetically tractable (Donohoue, Barrangou, & May, 2017).

## 3.2 Plant polyketides

Polyketides are a large class of secondary metabolites found in most plants. They can be further grouped into several subclasses like the chalcones, stilbenes, phloroglucinols, resorcinols, benzophenones, biphenyls, bibenzyls, chromones, acridones, pyrones, and curcuminoids (Abe & Morita, 2010). Polyketides have various functions in plants, such as pigmentation, UV protection, signaling, or plant defense (Lattanzio, Kroon, Quideau, & Treutter, 2008). Their common feature is that a type III polyketide synthase performs the committed step in their biosynthesis. These enzymes act as homodimers and homologs were found in all land plants with available genomic data. They condense various numbers of extender units, most commonly malonyl-CoA, onto an acyl-CoA starter unit to form a linear polyketide, which is then intramolecularly cyclized to form the core polyketide structure (Shimizu, Ogata, & Goto, 2017). Some examples of enzymes, using various starter units and numbers of condensation, are shown in Figure 3. Polyketides are an important part of the human diet. Certain compound families like proanthocyanidins are ubiquitously found in many dietary plants, while others are particularly abundant in certain foods (e.g. dihydrochalcones in apples or isoflavones in soy) (Cheynier, 2012). Various health benefits have been linked with their consumption (K. Pandey & Rizv, 2009). Well researched examples are the metabolic health promoting effects of resveratrol (Schrauwen & Timmers, 2014), the anti-cancer effects of curcumin (Shanmugam et al., 2015), or the health promoting effects of soy isoflavones in post-menopausal women (Messina, 2014). They also find other applications in various industries: anthocyanins are used as natural colorants in

many different applications (Cortez, Luna-Vital, Margulis, & Gonzalez de Mejia, 2017), homoeriodictyol can be used as bitterness masking agent (Ley, Krammer, Reinders, Gatfield, & Bertram, 2005), and resveratrol can be polymerized into a fire-resistant, light, and halogen free composite material (Evolva, 2016).

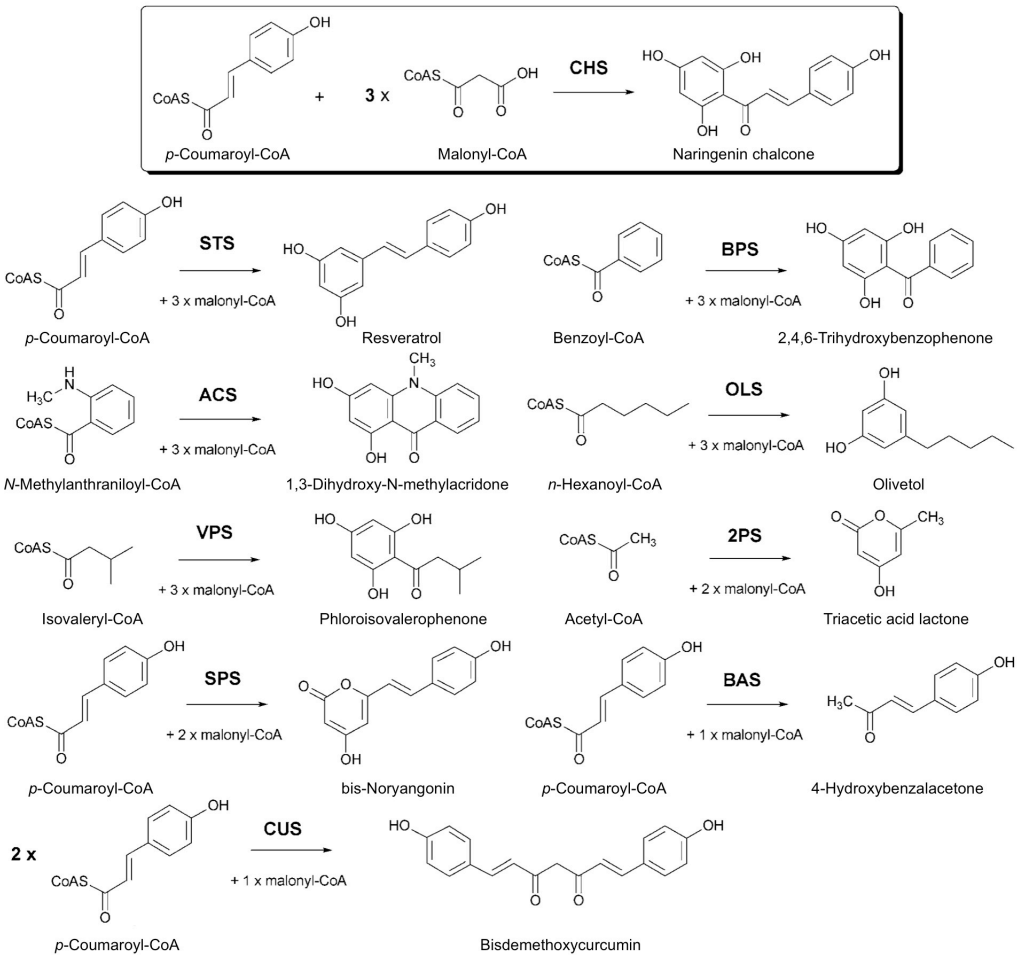


**Figure 3.** Type III polyketide synthase reactions catalyzed by various enzymes. ACS, acridone synthase; BAS, benzalacetone synthase; BBS, bibenzyl synthase; BPS, benzophenone synthase; CHS, chalcone synthase; CUS, curcuminoid synthase; OLS, olivetol synthase; 2PS, 2-pyrone synthase; SPS, styrylpyrone synthase; STS, stilbene synthase; VPS, phlorisovalerophenone synthase. Adapted from (Abe & Morita, 2010)

Since the first report of heterologous naringenin production in *E. coli* in 2003 (Hwang, Kaneko, Ohnishi, & Horinouchi, 2003), there have been many academic studies on heterologous production of flavonoids, stilbenoids, and curcuminoids using unicellular hosts, particularly *E. coli* and *S. cerevisiae*. These efforts have recently been excellently reviewed (Gottardi, Reifenrath, Boles, & Tripp, 2017; R. P. Pandey, Parajuli, Koffas, & Sohng, 2016; Wang, Guleria, Koffas, & Yan, 2016). In contrast,

only one commercial biotechnological process for production of plant polyketides is publicly disclosed: production of the Veri-te™ branded resveratrol by an engineered yeast strain, developed by the Swiss company Evolva (Evolva, n.d.).

## 3.3 Dihydrochalcones

### 3.3.1 Introduction to dihydrochalcones

Since phlorizin was identified in apples as the first dihydrochalcone in 1835 (Petersen, 1835), there has been significant interest in this class of compounds, accompanied by scientific research into their potential benefits for humans. While dihydrochalcones have long been thought to be restricted to plants belonging to about 30 plant families (Ninomiya & Koketsu, 2013), they were more recently found in significant amounts in grapes and raspberries, and with the development of novel and more sensitive analytical methods it is becoming obvious that they might be more widespread (Carvalho et al., 2013; Vrhovsek et al., 2012). Apples however remain unique in that they accumulate dihydrochalcones to very high concentrations of up to 14% dry weight in leaves (Gosch, Halbwirth, & Stich, 2010). Although knowledge about the functional role of dihydrochalcones *in planta* is limited, many of these structures have received attention for other reasons, mostly relating to a variety of human health and food applications (Rozmer & Perjési, 2016). Three of the most prominent examples of active dihydrochalcones described in literature are briefly outlined here. Phlorizin was found to be a hypoglycemic agent, acting by inhibiting SGLT1 and SGLT2, the human glucose transporters involved in intestinal glucose absorption and renal glucose reabsorption (Ehrenkranz, Lewis, Kahn, & Roth, 2005). It was later used as a blueprint for the development of over ten synthetic antidiabetic drugs (Chao, 2014), three of which have been approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) (Scheen, 2015). Aspalathin and nothofagin, which are naturally found in rooibos (*Aspalathus linearis*), exhibit strong antioxidant activity (Snijman et al., 2009) and are absorbed by the human body as intact glycosides due to the metabolic stability of their C-C-glycosidic bond (Breiter et al., 2011). Naringin dihydrochalcone (NDC) and neohesperidin dihydrochalcone, which can be chemically synthesized from citrus flavanones, are sweeteners with one and twenty times the sweetness of saccharin on a molar basis,

respectively (Horowitz & Gentili, 1969). In Europe, neohesperidin dihydrochalcone is approved as the food additive E959 (Janvier, Goscinny, Le Donne, & Van Loco, 2015). Chemically, the dihydrochalcones comprise a 1,3-diphenylpropan-1-one skeleton. They are further functionalized, mainly on the two aromatic rings, by hydroxylation, methylation, prenylation, glycosylation, and/or polymerization. Over 200 structurally different dihydrochalcones have been identified to date (Rozmer & Perjési, 2016).

### 3.3.2 Biosynthesis of dihydrochalcones

The proposed biosynthesis of dihydrochalcones in plants is shown in Figure 4. The early pathway from phenylalanine to *p*-coumaroyl-CoA is catalyzed by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate-CoA ligase (4CL). These steps are shared with the biosynthesis of other polyketides, such as flavonoids or stilbenoids, and have been thoroughly studied and characterized (Gosch, Halbwirth, & Stich, 2010). The action of a double bond reductase (DBR) to form *p*-dihydrocoumaroyl-CoA from *p*-coumaroyl-CoA appears to be required to initiate the biosynthesis of dihydrochalcones (Dare, Tomes, Cooney, Greenwood, & Hellens, 2013; Gosch, Halbwirth, Kuhn, Miosic, & Stich, 2009). Three different enzymes from apple (*Malus* x *domestica*) have recently been suggested to catalyze this reaction *in planta* (Dare, Tomes, Cooney, et al., 2013; Ibdah et al., 2014). Phloretin, the first committed dihydrochalcone, is then formed by decarboxylative condensation with three units of malonyl-CoA and a subsequent cyclisation, all catalyzed by chalcone synthase (CHS). CHS was found to be shared between the flavonoid and dihydrochalcone pathways in apple (Gosch et al., 2009). A range of enzymes responsible for further decoration of dihydrochalcones has been identified. Several UDP-dependent-glycosyltransferases (UGTs) from apple, pear, and carnation glycosylate the 2'-hydroxygroup of phloretin to form phlorizin (Gosch, Halbwirth, Schneider, Hölscher, & Stich, 2010; Jugdé, Nguy, Moller, Cooney, & Atkinson, 2008; Werner & Morgan, 2009), two UGTs from rice and buckwheat C-glycosylate the 3'-position of phloretin to form nothofagin (Brazier-Hicks et al., 2009; Ito, Fujimoto, Shimosaka, & Taguchi, 2014), *MdPh-4'-OGT* from *Malus* x *domestica* glycosylates the 4'- hydroxygroup of phloretin to form trilobatin (Yahyaa et al., 2016), and chalcone 3-hydroxylase (CH3H) from *Cosmos sulphureus* hydroxylates phloretin to form 3-hydroxyphloretin in yeast and in plants (Hutabarat et al., 2016) (Figure 4B).

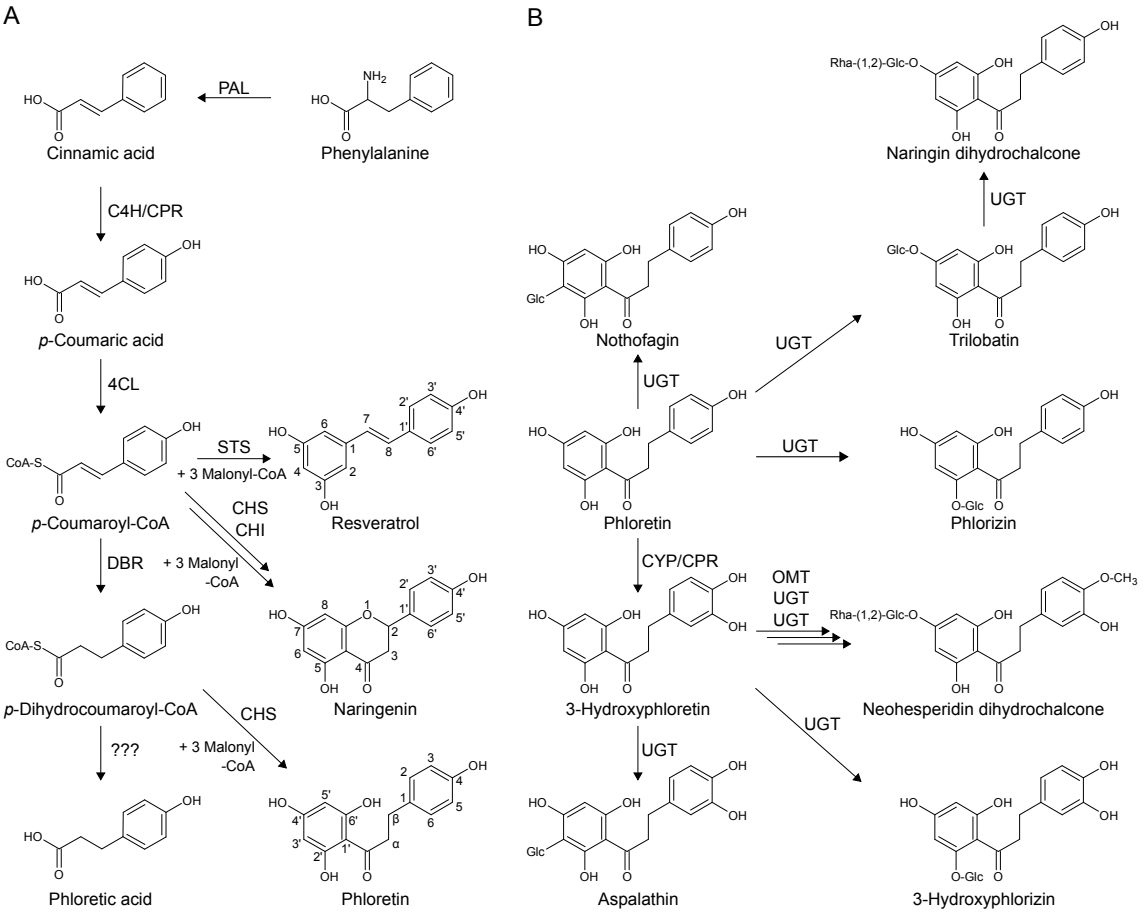


**Figure 4.** (A) The biosynthetic pathway to the first committed dihydrochalcone (phloretin), flavonoid (naringenin), and stilbenoid (resveratrol) from phenylalanine and malonyl-CoA is shown. (B) Depicted are proposed biosynthetic routes to several hydroxylated, glycosylated, and methylated dihydrochalcone derivatives. CHI, chalcone isomerase; CHS, chalcone synthase; CPR, cytochrome P450 reductase; CYP, cytochrome P450; C4H, cinnamate 4-hydroxylase; DBR, double bond reductase; OMT, O-methyltransferase; PAL, phenylalanine ammonia lyase; STS, stilbene synthase; UGT, UDP-dependent-glycosyltransferase; 4CL, 4-coumarate-CoA ligase.

### 3.3.3 Metabolic engineering of microorganisms for production of dihydrochalcones

Metabolic engineering of dihydrochalcones in microorganisms has been the subject of only three studies. Two of these studies (Watts, Lee, & Schmidt-Dannert, 2004; Werner, Chen, Jiang, & Morgan, 2010) expressed 4CL and CHS enzymes in *E. coli* and *S. cerevisiae*, respectively, and produced phloretin by feeding phloretic acid. This strategy circumvents the critical DBR step needed to generate the starter molecule for dihydrochalcone biosynthesis. In another study, a CHI and an enoate reductase from *Eubacterium ramulus* was expressed in *E. coli* and the compounds phloretin, 3-hydroxyphloretin, and homoeriodictyol dihydrochalcone were produced by feeding

naringenin, eriodictyol, and homoeriodictyol, respectively (Gall et al., 2014). However, besides the high cost of these precursors, this strategy additionally required anaerobic cultivation to achieve sufficient enzymatic activity.

## 3.4 Anthocyanins

### 3.4.1 Introduction to anthocyanins

Anthocyanins, together with other flavonoids, belong to a large group of diverse secondary metabolites found in almost the entire plant kingdom. They are probably best known for the colors they confer to plant tissues, in particular the red, orange, purple, and blue colors of flower petals, fruits, and leaves. While attraction of pollinators is the most obvious function of anthocyanins *in planta*, they have also been linked to shielding of abiotic stresses (UV-B radiation, temperature variation, and mineral stress) and to active defensive roles against pathogens, insects, and herbivores (Andersen & Jordheim, 2010).

From a human perspective, anthocyanins have found applications as colorants in the food and beverage industry, and the demand for such natural colors are expected to increase (Cortez et al., 2017). In addition, it has become clear that anthocyanins may have benefits in human health. Numerous studies are currently being conducted to elucidate their potential health promoting effects, ranging from antioxidant activity, cardiovascular protection, neuroprotection, vision improvement, antidiabetic properties, anti-inflammatory effects, cancer protection to antimicrobial activity (Pojer, Mattivi, Johnson, & Stockley, 2013).

Although derived from the same biosynthetic plant pathway the anthocyanins are a quite diverse class of compounds, with complex patterns of glycosylation and acylation. To date, 644 different structure elucidated anthocyanins have been isolated from various plants (Andersen & Jordheim, 2010).

### 3.4.2 Biosynthesis of anthocyanins

The biosynthesis of anthocyanins in plants proceeds via the general flavonoid pathway (Figure 5).

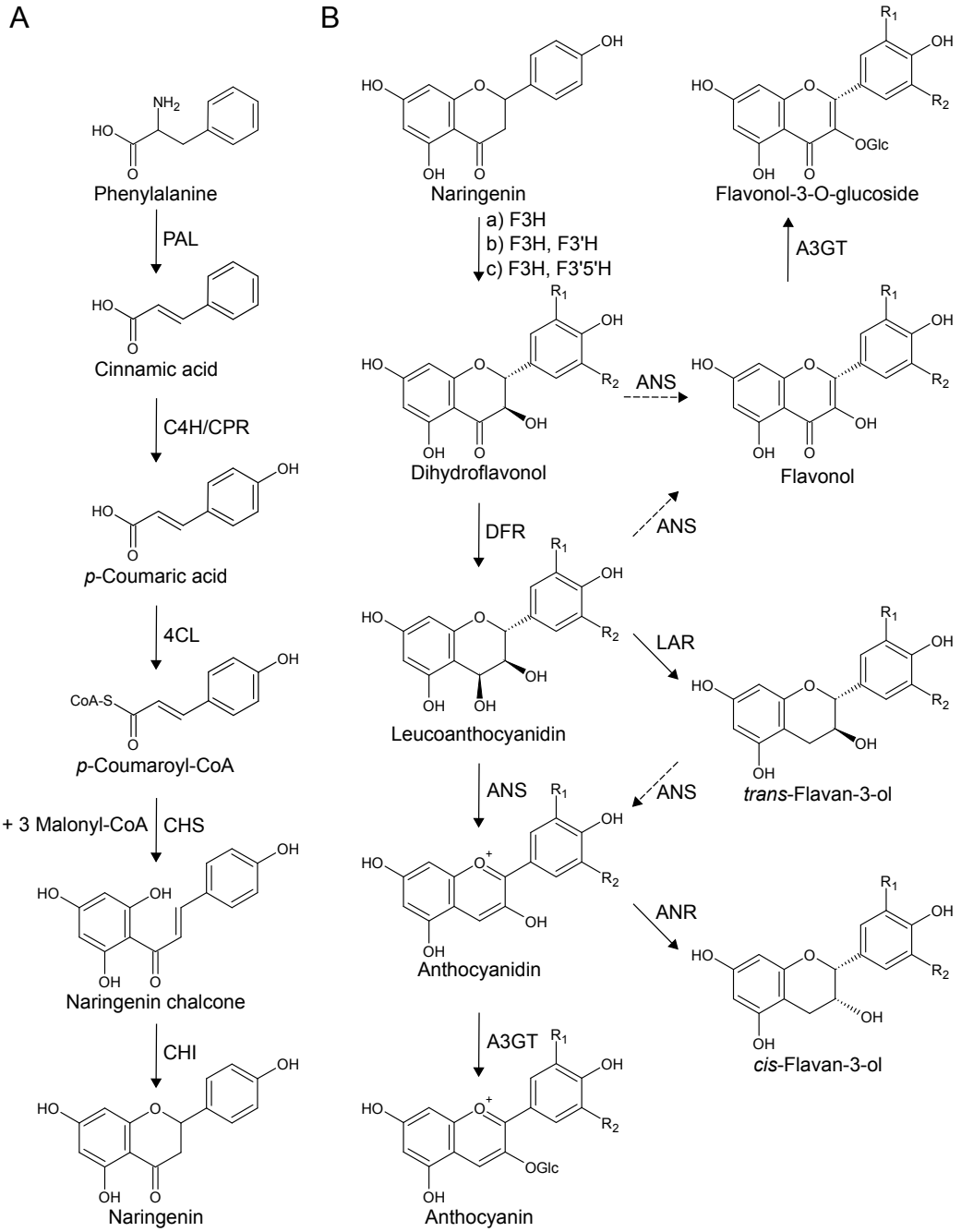


**Figure 5.** (A) Pathway from phenylalanine and malonyl-CoA to naringenin. (B) Linear pathway from naringenin to anthocyanins, as well as the deviation via *trans*-flavan-3-ols, which was successfully incorporated in *E. coli*, and the flavonol by-products derived from ANS side activities. Names of compounds with different hydroxylation patterns can be found in Table 4. ANS: anthocyanidin synthase; ANR: anthocyanidin reductase; A3GT: anthocyanidin 3-O-glycosyl transferase; CHI: chalcone isomerase; CHS: chalcone synthase; C4H: cinnamate 4-hydroxylase; CPR: cytochrome P450 reductase; DFR: dihydroflavonol-4-reductase; F3H: flavanone 3-hydroxylase; F3'H: flavonoid-3'-hydroxylase; F3'5'H: flavonoid-3',5'-hydroxylase; LAR: leucoanthocyanidin reductase; PAL: phenylalanine ammonia lyase; 4CL: 4-coumarate-CoA ligase

Like for dihydrochalcones, the activated phenylpropanoic acid *p*-coumaroyl-CoA is produced from the aromatic amino acid phenylalanine by a sequence of three enzymes: phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate-CoA ligase (4CL). Chalcone synthase (CHS) condenses *p*-coumaroyl-CoA with three malonyl-CoA extender units and cyclizes the molecule to form naringenin chalcone, which is then isomerized to naringenin by chalcone isomerase (CHI). Flavanone 3-hydroxylase (F3H) catalyzes the hydroxylation of naringenin at the 3-position to yield dihydrokaempferol, a dihydroflavonol. F3H belongs to the 2-oxoglutarate-dependent dioxygenase (2ODD) family. Naringenin and dihydrokaempferol can be hydroxylated by the cytochrome P450 (CYP) enzymes flavonoid-3'-hydroxylase (F3'H) or flavonoid-3',5'-hydroxylase (F3'5'H) to form eriodictyol and 5,7,3',4',5'-pentahydroxyflavanone, or dihydroquercetin and dihydromyricetin, respectively. Presence of these two enzymes determines the hydroxylation pattern of the *p*-coumaroyl-CoA derived B ring of both flavonoids and anthocyanins.

**Table 4.** Names and abbreviations of compound classes and compounds in the pathway from naringenin to the three main anthocyanins.

| **Compound class** | **$R_1$=$R_2$=H** | **$R_1$=OH, $R_2$=H** | **$R_1$=$R_2$=OH** |
|---|---|---|---|
| Flavanone | Naringenin | Eriodictyol | 5,7,3',4',5'-Penta-hydroxyflavanone |
| Dihydroflavonol | Dihydrokaempferol | Dihydroquercetin | Dihydromyricetin |
| Flavonol | Kaempferol | Quercetin | Myricetin |
| Flavonol-3-O-glucoside | Kaempferol-3-O-glucoside | Quercetin-3-O-glucoside | Myricetin-3-O-glucoside |
| Leucoanthocyanidin | Leucopelargonidin | Leucocyanidin | Leucodelphinidin |
| Anthocyanidin | Pelargonidin | Cyanidin | Delphinidin |
| Anthocyanin | Pelargonidin-3-O-glucoside | Cyanidin-3-O-glucoside | Delphinidin-3-O-glucoside |
| *trans*-Flavan-3-ol | Afzelechin | Catechin | Gallocatechin |
| *cis*-Flavan-3-ol | Epiafzelechin | Epicatechin | Epigallocatechin |

Dihydroflavonol-4-reductase (DFR) then catalyzes the reduction of dihydroflavonols to the corresponding leucoanthocyanidins, which are subsequently oxidized to the corresponding anthocyanidins by anthocyanidin synthase (ANS, also called leucoanthocyanidin dioxygenase (LDOX)), also belonging to the 2ODD family.

This step has been problematic both *in vitro* and in *E. coli*, where ANS converts leucoanthocyanidins primarily into flavonols instead of anthocyanidins, probably due to a second oxidation by ANS (Turnbull et al., 2003; Yan, Li, & Koffas, 2008). Both leucoanthocyanidin and anthocyanidin are relatively unstable intermediates, and in order to form the more stable anthocyanins, anthocyanidins are 3-O-glucosylated by the action of a UDP-glucose dependent anthocyanidin 3-O-glycosyl transferase (A3GT) (Tanaka, Sasaki, & Ohmiya, 2008).

The three basic anthocyanins, pelargonidin-3-O-glucoside, cyanidin-3-O-glucoside, and delphinidin-3-O-glucoside differ by the number of hydroxyl-groups on the B-ring. These hydroxyl-groups are in some cases further O-methylated to yield peonidin, petunidin, malvidin, and other less commonly found structures (Davies, 2009). Further modifications include glycosylation at one or more hydroxyl-groups and in some cases further glycosylation and/or acylation of these sugars (Sasaki, Nishizaki, Ozeki, & Miyahara, 2014; Tanaka et al., 2008). The most commonly found sugar moieties are glucose, rhamnose, galactose, xylose, arabinose, and glucuronic acid. Common acyl groups include the dicarboxylic acid malonic acid, various hydroxycinnamic acids (*p*-coumaric-, caffeic-, ferulic-, sinapic-, and 3,5-dihydrocinnamic acids), as well as hydroxybenzoic acids (*p*-hydroxybenzoic- and gallic acids) (Andersen & Jordheim, 2010). Enzymes catalyzing many of these decoration reactions have been discovered (Yonekura-Sakakibara, Nakayama, Yamazaki, & Saito, 2009).

### 3.4.3 Transport of anthocyanins

In plants, anthocyanins usually accumulate in the vacuole. Both the vacuolar pH and the presence of co-pigments play a role in stability and color of the anthocyanins (Passeri, Koes, & Quattrocchio, 2016; J. Zhao & Dixon, 2010). In various plants, transporters belonging to the ABC or MATE families were suggested to be responsible for this accumulation. ABC transporters use the energy of ATP hydrolysis and seem to be dependent on co-transport of glutathione, while MATE transporters use a proton gradient as driving force (Francisco et al., 2013; Marinova et al., 2007; J. Zhao, 2015; J. Zhao & Dixon, 2010). By binding to anthocyanins, glutathione-S-transferases (GSTs) were suggested as carrier proteins, which present anthocyanins to the transporters (Mueller, Goodman, Silady, & Walbot, 2000; Sun, Li, & Huang, 2012). Plants mutated in transporters or GSTs were shown to accumulate only a fraction of the anthocyanins compared to the wild type, and therefore vacuolar transport of anthocyanins is thought

to be essential for their production in plants (Alfenito et al., 1998; Goodman, Casati, & Walbot, 2004; Kitamura, Oono, & Narumi, 2016; Kitamura, Shikazono, & Tanaka, 2004; Sun et al., 2012; Yamazaki et al., 2008). However, it is still a matter of debate how these different transport systems may interact and complement each other (J. Zhao, 2015).

### 3.4.4 Metabolic engineering of microorganisms for production of anthocyanins

A series of studies investigated conversion of flavonoid precursors to several glycosylated, methylated, and acylated anthocyanins by engineered *E. coli*. These efforts have recently been reviewed (Zha & Koffas, 2017). Selection of optimal enzymes, as well as optimization of cofactors, culturing conditions, and expression conditions has resulted in cyanidin-3-O-glucoside production from flavan-3-ol precursors of up to 350 mg/l of, while production from flavanones never exceeded 2.07 mg/l (Chemler & Koffas, 2008; Cress et al., 2017; C. G. Lim et al., 2015; Yan, Chemler, Huang, Martens, & Koffas, 2005; Yan et al., 2008; Zha & Koffas, 2017). More recently, production of up to 9.5 mg/l pelargonidin-3-O-glucoside from glucose was shown by a synthetic polyculture approach of four engineered *E. coli* strains, each optimized for expression of a module of the complete pathway (Jones et al., 2017).

## 3.5 Scope of the thesis

This thesis aimed at extending the range of plant polyketides attainable by metabolic engineering of the yeast *S. cerevisiae* with a focus on two compound classes. The dihydrochalcones contain several commercially relevant compounds with sweet, antidiabetic, or antioxidant properties. The anthocyanins are already in use as natural colorants in the food and beverage industries. Proof of concepts for *de novo* biosynthesis of compounds within these two classes should highlight the potential of yeast as cell factory for polyketides. Therefore, this thesis should lay the foundation and point towards the next steps required for the development of a more sustainable production process of these compounds.

# 4 Materials and Methods

## 4.1 Chemicals

Unless stated otherwise, chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Authentic standards of cyanidin, cyanidin-3-O-glucoside, cyanidin-3,5-O-diglucoside, delphinidin, delphinidin-3-O-glucoside, delphinidin-3,5-O-diglucoside, myricetin-3-O-glucoside, pelargonidin, pelargonidin-3-O-glucoside, pelargonidin-3,5-O-diglucoside, phloretin, and trilobatin were purchased from Extrasynthese (Genay, France). Authentic standards of 3-hydroxyphloretin and dihydrokaempferol were purchased from PlantMetaChem (Giessen, Germany). Authentic standards of gallocatechin and pinocembrin dihydrochalcone were purchased from Toronto Research Chemicals (North York, Canada) and AnalytiCon (Potsdam, Germany), respectively. Nothofagin and afzelechin were kindly provided by Dr. Alexander Gutmann (Graz University, Austria) and Dr. Stefan Martens (Fondazione Edmund Mach, San Michele all'Adige, Italy), respectively.

## 4.2 Plasmids and enzymes

*E. coli* XL10 Gold (Agilent, Santa Clara, California, USA) cells were used for subcloning of genes. Table 5 shows all genes used in this study, as well as the vector backbones they were cloned into. Coding sequences for selected enzymes were cloned into expression cassettes of plasmids pEVE2176 to pEVE2181 for assembly by *in vivo* homologous recombination (see 4.4) or into yeast expression plasmids pEVE2157, pEVE2159, and pEVE2164, which are based on the pRS series of shuttle plasmids (Sikorski & Hieter, 1989). Generally, restriction enzyme and ligation based cloning with *HindIII* HF, *SacII*, and T4 DNA ligase (all NEB, Ipswich, Massachusetts, USA) according to standard protocols was used for plasmid construction (Green & Sambrook, 2012). Genes containing internal *HindIII* or *SacII* sites were cloned by In-Fusion (Takara, Kyoto, Japan) according to the manufacturer's instructions. *E. coli* was grown in LB medium prepared with 25 g/l of LB broth (Miller) and supplemented with 100 µg/l ampicillin for amplification of plasmids.

*S. cerevisiae* codon optimized genes were manufactured by GeneArt (ThermoFisher, Waltham, Massachusetts, USA), except genes in pDHC25 and pDHC26, which were obtained by site directed mutagenesis of pDHC24 using overlap extension PCR with primers DHPR1 to DHPR10 (Table 6) according to a standard protocol (Heckman & Pease, 2007). During synthesis or PCR, all genes were provided at the 5' end with an AAGCTTAAA DNA sequence comprising a *HindIII* site and a Kozak sequence and at the 3' end a GGCGCC DNA sequence comprising a *SacII* restriction site.

**Table 5.** List of genes used in this work and plasmid backbones into which they were cloned.

| Plasmid name | Gene | Backbone | Codon optimized | Organism | NCBI protein accession number |
|---|---|---|---|---|---|
| pDHC1 | *AtPAL2* | pEVE2179 | yes | *Arabidopsis thaliana* | NP_190894.1 |
| pDHC2 | *AmC4H* | pEVE2180 | yes | *Ammi majus* | AAO62904.1 |
| pDHC3 | *ScCPR1* | pEVE2181 | no | *S. cerevisiae* | NP_011908.1 |
| pDHC4 | *At4CL2* | pEVE2178 | no | *A. thaliana* | NP_188761.1 |
| pDHC5 | *HaCHS* | pEVE2176 | yes | *Hypericum androsaemum* | AAG30295.1 |
| pDHC6 | *ScDFG10* | pEVE2177 | no | *S. cerevisiae* | NP_012215.1 |
| pDHC7 | *ScTSC13* | pEVE2177 | no | *S. cerevisiae* | NP_010269.1 |
| pDHC8 | *KlTSC13* | pEVE2177 | yes | *Kluyveromyces lactis* | XP_452392.1 |
| pDHC9 | *AtECR* | pEVE2177 | yes | *A. thaliana* | NP_191096.1 |
| pDHC10 | *GhECR2* | pEVE2177 | yes | *Gossypium hirsutum* | NP_001314306.1 |
| pDHC11 | *MdECR* | pEVE2177 | yes | *M.* x *domestica* | XP_008382818.1 |
| pDHC12 | *MdENRL3* | pEVE2177 | yes | *M.* x *domestica* | NP_001280847.1 |
| pDHC13 | *MdENRL5* | pEVE2177 | yes | *M.* x *domestica* | NP_001281005.1 |
| pDHC14 | *MdHCDBR* | pEVE2177 | yes | *M.* x *domestica* | XP_008367739.1 |
| pDHC15 | *ErERED* | pEVE2177 | yes | *Eubacterium ramulus* | AGS82961.1 |
| pDHC16 | *RiZS1* | pEVE2177 | yes | *Rubus idaeus* | AEL78825.1 |
| pDHC17 | *PcCHS* | pEVE2176 | yes | *Petroselinum crispum* | CAA24779.1 |
| pDHC18 | *PhCHS* | pEVE2176 | yes | *Petunia* x *hybrida* | CAA32731.1 |
| pDHC19 | *HvCHS1* | pEVE2176 | yes | *Hordeum vulgare* | CAA41250.1 |
| pDHC20 | *HvCHS2* | pEVE2176 | yes | *H. vulgare* | CAA70435.1 |
| pDHC21 | *SbCHS* | pEVE2176 | yes | *Scutellaria baicalensis* | BAB03471.1 |
| pDHC22 | *MdCHS1* | pEVE2176 | no | *M.* x *domestica* | NP_001306186.1 |
| pDHC23 | *MdCHS2* | pEVE2176 | no | *M.* x *domestica* | NP_001306181.1 |
| pDHC24 | *MdUGT88F1* | pEVE2176 | yes | *M.* x *domestica* | ACZ44840.1 |
| pDHC25 | *MdUGT88A1* | pEVE2176 | yes | *M.* x *domestica* | ABY73540.1 |
| pDHC26 | *PcUGT88F2* | pEVE2176 | yes | *Pyrus communis* | ACZ44838.1 |
| pDHC27 | *DcGT4* | pEVE2176 | yes | *Dianthus caryophyllus* | BAD52006.1 |
| pDHC28 | *OsCGT* | pEVE2176 | yes | *Oryza sativa subsp. jap.* | ABC94602.1 |
| pDHC29 | *AtUGT73B2* | pEVE2178 | yes | *A. thaliana* | NP_567954.1 |
| pDHC30 | *AtUGT76D1* | pEVE2178 | no | *A. thaliana* | NP_180216.1 |
| pDHC31 | *AtUGT84B1* | pEVE2178 | no | *A. thaliana* | NP_179907.1 |
| pDHC32 | *Cm1,2RHAT* | pEVE2179 | yes | *Citrus maxima* | AAL06646.2 |
| pDHC33 | *AtRHM2* | pEVE2180 | no | *A. thaliana* | NP_564633.2 |
| pDHC34 | *OsF3'H* | pEVE2177 | yes | *O. sativa subsp. jap.* | XP_015613041.1 |
| pDHC35 | *PhF3'H* | pEVE2177 | yes | *P.* x *hybrida* | AAD56282.1 |

| | | | | | |
|---|---|---|---|---|---|
| pDHC36 | *PfF3'H* | pEVE2177 | yes | *Perilla frutescens* | BAB59005.1 |
| pDHC37 | *AcF3'H* | pEVE2177 | yes | *Allium cepa* | AAS48419.1 |
| pDHC38 | *MdF3'H1* | pEVE2177 | no | *M.* x *domestica* | ACR14867.1 |
| pDHC39 | *MdF3'H2* | pEVE2177 | no | *M.* x *domestica* | XP_008374610.1 |
| pDHC40 | *CsCH3H* | pEVE2177 | yes | *C. sulphureus* | ACO35755.1 |
| pDHC41 | *AtATR1* | pEVE2178 | yes | *A. thaliana* | NP_194183.1 |
| pDHC42 | *CaER* | pEVE2177 | yes | *Clostridium acetobutylicum* | WP_010966642.1 |
| pANT1 | *MsCHI* | pEVE2177 | yes | *Medicago sativa* | AAB41524.1 |
| pANT2 | *VvF3'H* | pEVE2177 | yes | *Vitis vinifera* | CAI54278.1 |
| pANT3 | *PhF3'H* | pEVE2177 | yes | *P.* x *hybrida* | AAD56282.1 |
| pANT4 | *OsF3'H* | pEVE2177 | yes | *O. sativa subsp. jap.* | AEK31169.1 |
| pANT5 | *CrF3'5'H* | pEVE2177 | yes | *Catharanthus roseus* | CAA09850.1 |
| pANT6 | *PhF3'5'H* | pEVE2177 | yes | *P.* x *hybrida* | CAA80266.1 |
| pANT7 | *OhF3'5'H* | pEVE2177 | yes | *Osteospermum hybrid cul* | ABB43031.1 |
| pANT8 | *SlF3'5'H* | pEVE2177 | yes | *Solanum lycopersicum* | ACF32346.1 |
| pANT9 | *CiF3'5'H* | pEVE2177 | yes | *Cichorium intybus* | AGO03825.1 |
| pANT10 | *AtCPR1* | pEVE2178 | yes | *A. thaliana* | CAA46814.1 |
| pANT11 | *FaF3H* | pEVE2178 | yes | *Fragaria* x *ananassa* | AAU04791.1 |
| pANT12 | *MdF3H* | pEVE2178 | yes | *M.* x *domestica* | CAA49353.1 |
| pANT13 | *NtF3H* | pEVE2178 | yes | *Nicotiana tabacum* | BAF96938.1 |
| pANT14 | *AtDFR* | pEVE2179 | yes | *A. thaliana* | AAA32783.1 |
| pANT15 | *AaDFR* | pEVE2179 | yes | *Anthurium andraeanum* | AAP20866.1 |
| pANT16 | *PtDFR* | pEVE2179 | yes | *Populus trichocarpa* | EEE80032.1 |
| pANT17 | *IhDFR* | pEVE2179 | yes | *Iris hollandica* | BAF93856.1 |
| pANT18 | *MdDFR* | pEVE2179 | no | *M.* x *domestica* | AAD26204.1 |
| pANT19 | *VvLAR* | pEVE2180 | yes | *V. vinifera* | CAI26310.1 |
| pANT20 | *InANS* | pEVE2176 | yes | *Ipomoea nil* | BAB71806.1 |
| pANT21 | *GhANS* | pEVE2176 | yes | *Gerbera* x *hybrida* | AAY15743.2 |
| pANT22 | *MdANS* | pEVE2176 | yes | *M.* x *domestica* | BAB92998.1 |
| pANT23 | *PhANS* | pEVE2176 | yes | *P.* x *hybrida* | P51092.1 |
| pANT24 | *FaANS* | pEVE2176 | yes | *F.* x *ananassa* | AAU12368.1 |
| pANT25 | *PcANS* | pEVE2176 | yes | *P. communis* | ABB70119.1 |
| pANT26 | *IbANS* | pEVE2176 | yes | *Ipomoea batatas* | ADE08370.1 |
| pANT27 | *StANS* | pEVE2176 | yes | *Solanum tuberosum* | AEJ90548.1 |
| pANT28 | *MsANS* | pEVE2176 | yes | *Magnolia sprengeri* | AHU88620.1 |
| pANT29 | *GbANS* | pEVE2176 | yes | *Ginkgo biloba* | ACC66092.1 |
| pANT30 | *ZmANS* | pEVE2176 | yes | *Zea mays* | CAA39022.1 |
| pANT31 | *AtANS* | pEVE2176 | yes | *A. thaliana* | AAB09572.1 |
| pANT32 | *OsANS* | pEVE2176 | yes | *O. sativa subsp. indica* | CAA69252 |
| pANT33 | *AcANS* | pEVE2176 | yes | *A. cepa* | ABM66367.1 |
| pANT34 | *OsA3GT* | pEVE2164 | yes | *O. sativa subsp. jap.* | BAB68093.1 |
| pANT35 | *GtA3GT-1* | pEVE2164 | yes | *Gentiana triflora* | BAC54092.1 |
| pANT36 | *PhA3GT* | pEVE2164 | yes | *P.* x *hybrida* | BAA89008.1 |
| pANT37 | *AtA3GT* | pEVE2164 | yes | *A. thaliana* | CAC01718.1 |
| pANT38 | *FaA3GT-1* | pEVE2164 | yes | *F.* x *ananassa* | AAU09442.1 |
| pANT39 | *VvA3GT* | pEVE2164 | yes | *V. vinifera* | AAB81682.1 |
| pANT40 | *FaA3GT-2* | pEVE2164 | yes | *F.* x *ananassa* | AAU12366.1 |
| pANT41 | *DcA3GT* | pEVE2164 | yes | *D. caryophyllus* | BAD52003.1 |
| pANT42 | *GtA3GT-2* | pEVE2164 | yes | *G. triflora* | BAA12737.1 |
| pANT43 | *FaA3GT-2* | pEVE2177 | yes | *F.* x *ananassa* | AAU12366.1 |
| pANT44 | *DcA3GT* | pEVE2177 | yes | *D. caryophyllus* | BAD52003.1 |
| pGST1 | *ZmBZ2* | pEVE2157 | yes | *Z. mays* | CAA57496 |

| | | | | | |
|---|---|---|---|---|---|
| pGST2 | *PhAN9* | pEVE2157 | yes | *P. x hybrida* | CAA68993 |
| pGST3 | *AtTT19* | pEVE2157 | yes | *A. thaliana* | NP_197224 |
| pGST4 | *AtTT19-3* | pEVE2157 | yes | *A. thaliana* | BAD89984.1 |
| pGST5 | *AtTT19-4* | pEVE2157 | yes | *A. thaliana* | NP_197224 (W205L) |
| pGST6 | *VvGST1* | pEVE2157 | yes | *V. vinifera* | AAN85826.1 |
| pGST7 | *VvGST2* | pEVE2157 | yes | *V. vinifera* | ABK81651.1 |
| pGST8 | *VvGST3* | pEVE2157 | yes | *V. vinifera* | ABO64930.1 |
| pGST9 | *VvGST4* | pEVE2157 | yes | *V. vinifera* | AAX81329 |
| pGST10 | *VvGST5* | pEVE2157 | yes | *V. vinifera* | ABL84692.1 |
| pGST11 | *PfGST1* | pEVE2157 | yes | *P. fructescens* | BAG14300 |
| pGST12 | *CsGST3* | pEVE2157 | yes | *Cyclamen spp* | BAM14584 |
| pGST13 | *TaGSTL1* | pEVE2157 | yes | *Triticum aestivum* | CAA76758.1 |
| pGST14 | *AtTT12* | pEVE2159 | yes | *A. thaliana* | NP_191462 |
| pGST15 | *SlMTP77* | pEVE2159 | yes | *S. lycopersicum* | AAQ55183 |
| pGST16 | *VvAM1* | pEVE2159 | yes | *V. vinifera* | ACN88706 |
| pGST17 | *VvAM3* | pEVE2159 | yes | *V. vinifera* | ACN91542 |
| pGST18 | *MtMATE1* | pEVE2159 | yes | *Medicago truncatula* | ADV04045 |
| pGST19 | *BnTT12-1* | pEVE2159 | yes | *Brassica napus* | ACJ36209 |
| pGST20 | *MdMATE1* | pEVE2159 | yes | *M.* x *domestica* | ADO22710 |
| pGST21 | *MdMATE2* | pEVE2159 | yes | *M.* x *domestica* | ADO22712 |
| pGST22 | *VvMATE1* | pEVE2159 | yes | *V. vinifera* | XP_002282932 |
| pGST23 | *VvMATE2* | pEVE2159 | yes | *V. vinifera* | XP_002282907 |
| pGST24 | *GaTT12a* | pEVE2159 | yes | *Gossypium arboretum* | AGC55236 |
| pGST25 | *ZmMRP3* | pEVE2159 | yes | *Z. mays* | AAT37905 |
| pGST26 | *VvABCC1* | pEVE2159 | yes | *V. vinifera* | AGC23330 |
| pGST27 | *DvA3GMAT* | pEVE2176 | yes | *Dahlia variabilis* | AAO12206 |
| pGST28 | *VaA5GT* | pEVE2180 | yes | *Vitis amurensis* | AHL68667.1 |
| pGST29 | *NsA3GRT* | pEVE2178 | yes | *Nierenbergia ssp.* | BAC10994.1 |
| pGST30 | *CtA3G3'5'GT* | pEVE2179 | yes | *Clitoria ternatea* | BAF49289 |

Genes in pDHC3, pDHC6, and pDHC7 were amplified by PCR (Q5 DNA polymerase, New England Biolabs) with primers DHPR11 to DHPR16 (Table 6) from a colony of *S. cerevisiae* S288C after lysis in 30 µl 0.2% SDS at 95°C for 5 minutes and clarification at 14000g for 5 minutes. For the genes in pDHC6 and pDHC7, restriction sites and Kozak sequence, as described above, were added during PCR. *ScCPR1* in pDHC3 was initially cloned with *SpeI* and *XhoI*, because it contained an internal *SacII* site. This site was then removed with a silent mutation (c519t) introduced by inverse PCR using primers DHPR17 and DHPR18 according to standard protocols (Green & Sambrook, 2012). For genes in pDHC4, pDHC22, pDHC23, pDHC30, pDHC31, pDHC33, pDHC38, pDHC39, and pANT18, RNA was extracted from *A. thaliana* leaves or *M.* x *domestica* var. Golden Delicious apple peel with the RNeasy kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. First strand cDNA was synthesized with the Mint-2 cDNA synthesis kit (Evrogen, Moscow, Russia) and genes were amplified by PCR (Q5 DNA polymerase) with primers DHPR19 to DHPR34 or

AcnPR1 to AcnPR6 (Table 6). For genes in pDHC4, pDHC22, pDHC23, pDHC30, pDHC31, pDHC33, pDHC38, and pDHC39, during PCR, an AAA Kozak sequence was added to the 5' end of the gene and 15 base pairs with homology to the plasmid backbone were added at the 5' and 3' end of the gene for cloning by In-Fusion into plasmid backbones linearized with *HindIII* and *SacII*. For *MdDFR* in pANT18, an overlap exchange PCR strategy using standard protocols (Heckman & Pease, 2007) was used to remove two internal *HindIII* sites of *MdDFR* with silent point mutations using primers AcnPR1 to AcnPR6, before cloning the open reading frame with *HindIII* and *SacII* (Table 6).

**Table 6.** List of primers used in this study.

| Primer name | Sequence | Description |
|---|---|---|
| DHPR1 | ACAAAAAGCTTAAAATGGGTGATG TCATTG | Forward primer for restriction enzyme based cloning of MdUGT88A1 and PcUGT88F2 |
| DHPR2 | AGCTACCGCGGTCAGGTAATGG | Reverse primer for restriction enzyme based cloning of MdUGT88A1 and PcUGT88F2 |
| DHPR3 | AGCAGCACCAGAAGTACAGAAGTA GTAGGTTGG | Reverse primer for site directed mutagenesis by overlap extension PCR of MdUGT88F1 to MdUGT88A1 (R139C) |
| DHPR4 | CCAACCTACTACTTCTGTACTTCTG GTGCTGCT | Forward primer for site directed mutagenesis by overlap extension PCR of *MdUGT88F1* to *MdUGT88A1* (R139C) |
| DHPR5 | ACCCAATTCAACCATAGCAACTAT ATGACCCAT | Reverse primer for site directed mutagenesis by overlap extension PCR of *MdUGT88F1* to *PcUGT88F2* (S18A) |
| DHPR6 | ATGGGTCATATAGTTGCTATGGTTG AATTGGGT | Forward primer for site directed mutagenesis by overlap extension PCR of *MdUGT88F1* to *PcUGT88F2* (S18A) |
| DHPR7 | AGAAAGCAGCCAAAACAGCAGCAC CAGAAGTATGGAAGTAGTAGGTT | Reverse primer for site directed mutagenesis by overlap extension PCR of *MdUGT88F1* to *PcUGT88F2* (R139H/I145V) |
| DHPR8 | AACCTACTACTTCCATACTTCTGGT GCTGCTGTTTTGGCTGCTTTCT | Forward primer for site directed mutagenesis by overlap extension PCR of *MdUGT88F1* to *PcUGT88F2* (R139H/I145V) |
| DHPR9 | CATCTTTCTCTCAAAGCTCTACCAC CTTCG | Reverse primer for site directed mutagenesis by overlap extension PCR of *MdUGT88F1* to *PcUGT88F2* (V449A) |
| DHPR10 | CCGAAGGTGGTAGAGCTTTGAGAG AAAGATG | Forward primer for site directed mutagenesis by overlap extension PCR of *MdUGT88F1* to *PcUGT88F2* (V449A) |
| DHPR11 | TACTAAGCTTACTAGTAAAATGCCG TTTGGAATAGACA | Forward primer for restriction enzyme based cloning of *ScCPR1* |
| DHPR12 | TCTCGAGTTTAAACCGCGGTTACCA GACATCTTCTTGGTA | Reverse primer for restriction enzyme based cloning of *ScCPR1* |
| DHPR13 | ACGTAAAGCTTAAAATGTACTTTGA | Forward primer for restriction enzyme based |

| | | |
|---|---|---|
| | TGAAGAACAATTGC | cloning of *ScDFG10* |
| DHPR14 | ACGTACCGCGGCTAGATTATAAAA GGTATTATGGCGTAG | Reverse primer for restriction enzyme based cloning of *ScDFG10* |
| DHPR15 | ACGTAAAGCTTAAAATGCCTATCA CCATAAAAAGC | Forward primer for restriction enzyme based cloning of *ScTSC13* |
| DHPR16 | ACGTACCGCGGTCAAAATACAAAT GGAATCAAGAATG | Reverse primer for restriction enzyme based cloning of *ScTSC13* |
| DHPR17 | P* CCCGCAGCGGAGAGATG | Forward primer for site directed mutagenesis by inverse PCR of *ScCPR1* (c519t) |
| DHPR18 | CGCTATCAGACTAGGCAAGCTC | Reverse primer for site directed mutagenesis by inverse PCR of *ScCPR1* (c519t) |
| DHPR19 | TAATTACAAAAAGCTAAAATGACG ACACAAGATGTG | Forward primer for In-Fusion cloning of *At4CL2* |
| DHPR20 | AGTTAAAAGCACTCCCTAGTTCATT AATCCATTTGCTAG | Reverse primer for In-Fusion cloning of *At4CL2* |
| DHPR21 | AACAAACAAAAAGCTAAAATGGTT ACAGTCGAGGAAG | Forward primer for In-Fusion cloning of *MdCHS1* |
| DHPR22 | GCCGTCGGACGTGCCTCAAGCCGTT AAACCCAC | Reverse primer for In-Fusion cloning of *MdCHS1* |
| DHPR23 | AACAAACAAAAAGCTAAAATGGTG ACCGTCGAAG | Forward primer for In-Fusion cloning of *MdCHS2* |
| DHPR24 | GCCGTCGGACGTGCCTCAAGCACC CACACTG | Reverse primer for In-Fusion cloning of *MdCHS2* |
| DHPR25 | TAATTACAAAAAGCTTAAAATGGC AGAGATTCGCC | Forward primer for In-Fusion cloning of *AtUGT76D1* |
| DHPR26 | AGTTAAAAGCACTCCGCGGTCATT GTTCGTCAATTTGCATC | Reverse primer for In-Fusion cloning of *AtUGT76D1* |
| DHPR27 | TAATTACAAAAAGCTAAAATGGGC AGTAGTGAGG | Forward primer for In-Fusion cloning of *AtUGT73B2* |
| DHPR28 | AGTTAAAAGCACTCCTTAGGCGATT GTGATATCACTAATGAAC | Reverse primer for In-Fusion cloning of *AtUGT73B2* |
| DHPR29 | TTAACTAAACAAGCTAAAATGGAT GATACTACGTATAAGCCAAAGAAC | Forward primer for In-Fusion cloning of *AtRHM2* |
| DHPR30 | AAGAGCGATTTGTCCTTAGGTTCTC TTGTTTGGTTCAAAGACG | Reverse primer for In-Fusion cloning of *AtRHM2* |
| DHPR31 | ATATAAAACAAAGCTAAAATGTTT GTTCTCATAGTCTTCACC | Forward primer for In-Fusion cloning of *MdF3'H1* |
| DHPR32 | AGACATAAGAGATCCTCAAGATGA TGATGCATTGTATGC | Reverse primer for In-Fusion cloning of *MdF3'H1* |
| DHPR33 | ATATAAAACAAAGCTAAAATGTTT GTTCTCATATTCTTCACCG | Forward primer for In-Fusion cloning of *MdF3'H2* |
| DHPR34 | AGACATAAGAGATCCTCAAGGTGA TGACGCATTATATG | Reverse primer for In-Fusion cloning of *MdF3'H2* |
| AcnPR1 | ACGTAAAGCTTAAAATGGATCCGA GTCCGAATCCG | Forward primer for restriction enzyme based cloning of *MdDFR* |
| AcnPR2 | AACAGTTCGAAAGCTAGTGTTCAC ATCCTCA | Reverse primer for restriction enzyme based cloning of *MdDFR* |

| | | |
|---|---|---|
| AcnPR3 | TATGGCTTCATCAAAACTTCCCTCA TCCGCC | Reverse primer for site directed mutagenesis by overlap extension PCR of *MdDFR* (c210t) |
| AcnPR4 | GGCGGATGAGGGAAGTTTTGATGA AGCCATA | Forward primer for site directed mutagenesis by overlap extension PCR of *MdDFR* (c210t) |
| AcnPR5 | TGAGGATGTGAACACTAGCTTTCG AACTGTT | Reverse primer for site directed mutagenesis by overlap extension PCR of *MdDFR* (t369a) |
| AcnPR6 | AACAGTTCGAAAGCTAGTGTTCAC ATCCTCA | Forward primer for site directed mutagenesis by overlap extension PCR of *MdDFR* (t369a) |

## 4.3 Yeast strains

*S. cerevisiae* strain BG (*MATα hoΔ0 his3Δ0 leu2Δ0 ura3Δ0 cat5Δ0::CAT5(I91M) mip1Δ0::MIP1(A661T) gal2Δ0::GAL2 sal1Δ0::SAL1*), which is a derivative of the S288C strain NCYC 3608 (NCYC, Norwich, United Kingdom), was used for all experiments in this work. The strain had been further modified in our labs. Briefly, the *LEU2* and *HIS3* open reading frames were deleted to create two additional auxotrophies for leucine and histidine, respectively. The *KanMX* cassette was excised by Cre-Lox recombination. The non-functional *gal2* gene was replaced with a functional allele from *S. cerevisiae* SK1 strain NCYC 3615 (NCYC) and the *sal1*, *mip1*, and *cat5* genes were engineered to reduce petite formation (Dimitrov, Brem, Kruglyak, & Gottschling, 2009). Generally, yeast cultures were grown in SC medium prepared with 1.47 g/l Synthetic Complete (Kaiser) Drop Out: Leu, His, Ura (Formedium, Hunstanton, United Kingdom), 6.7 g/l Yeast Nitrogen Base Without Amino Acids, 20 g/l D-(+)-Glucose, pH set to 5.8 with hydrochloric acid, and supplemented with 76 mg/l histidine, 380 mg/l leucine, and/or 76 mg/l uracil depending on the auxotrophies of the strains. A slow glucose release minimal medium as follows was used in some experiments: 19.2 g/l MES monohydrate, 3.5 g/l ammonium sulfate, 1.89 g/l citric acid monohydrate, 1.40 g/l potassium chloride, 1.77 g/l dipotassium hydrogenphosphate heptahydrate, 1.35 g/l magnesium sulfate heptahydrate, 1.35 g/l sodium chloride, 0.80 g/l calcium chloride dihydrate, 15 mg/l EDTA, 4.5 mg/l zinc sulfate heptahydrate, 0.3 mg/l cobalt(II) chloride hexahydrate, 1 mg/l manganese(II) chloride tetrahydrate, 0.3 mg/l copper(II) sulfate pentahydrate, 3 mg/l iron(II) sulfate heptahydrate, 0.4 mg/l sodium molybdate dihydrate, 1 mg/l boric acid, 0.05 mg/l biotin, 1 mg/l calcium pantothenate, 1 mg/l nicotinic acid, 25 mg/l inositol, 1 mg/l thiamine HCl, 1mg/l pyridoxine HCl, 0.2 mg/l para-aminobenzoic acid, pH set to pH 6.4 with potassium hydroxide for the normal

medium or to pH 4.0 with sulfuric acid for the low pH medium, and supplemented with 40 g/l polysaccharide (m2p-labs, Baesweiler, Germany) and 0.3% (v/v) Enzyme-Mix 100 U/ml (m2p-labs, Baesweiler, Germany).

## 4.4 Assembly of gene expression cassettes on multi-expression plasmids or into the genome by homologous recombination

Multiple expression cassettes were assembled into multi-expression plasmids or integrated into the genome with an *in vivo* homologous recombination technology (HRT) slightly modified from DNA assembler (Shao, Zhao, & Zhao, 2009). In contrast to the original version, the 60 base pair homology sequences used to assemble different cassettes were not added by PCR but were already present in HRT entry vectors. This allows indefinite reuse of cassettes after initial cloning and sequence verification of the respective genes. After cloning into these entry vectors, both expression cassettes and helper fragments required for assembly of HRT plasmids or integration into the genome are flanked by 60 base pair HRT tags named A, B, C, D, E, F, G, H, and Z, which are in turn flanked by *AscI* restriction sites for release of the tagged fragments. Table 7 lists all basic parts used in this work. Genes of interest were cloned into pEVE2176-2181 as shown in Table 5. For multi-expression HRT plasmid assembly 310 fmol each of the ZA and AB entry plasmids, containing all elements required for replication, maintenance, and selection, were combined with 460 fmol each of all other entry plasmids, containing the expression cassettes and the closing linker. The complete mixture was digested with *AscI* (NEB) in a 10 µl reaction, releasing all inserts from their vector backbone, followed by heat inactivation of the restriction enzyme at 80°C for 20 minutes. The whole reaction was used to transform *S. cerevisiae* with a standard LiAc transformation (Gietz & Schiestl, 2007). For integration of expression cassettes into the yeast genome the method was slightly adapted. The autonomous replication signal was omitted, and instead the ZA and AB helper fragments contained homologous sequences for integration into the genome and a selection marker nested between two LoxP sites. Additionally, the amount of DNA was scaled up by a factor of three compared to assembly of multi-expression plasmids in order to increase integration efficiency. All strains constructed in this work can be found in Table 8.

**Table 7.** List of basic HRT plasmids and expression plasmid used.

| Plasmid name | Content |
|---|---|
| pEVE4728 | *Z-tag pSC101 LEU2 A-tag* |
| pEVE4729 | *Z-tag pSC101 HIS3 A-tag* |
| pEVE4730 | *Z-tag pSC101 URA3 A-tag* |
| pEVE4745 | *Uptag XI-3 A-tag Z-tag Downtag XI-3* |
| pEVE22929 | *Uptag XI-2 A-tag Z-tag Downtag XI-2* |
| pEVE1968 | *A-tag ARS/CEN CmR B-tag* |
| pEVE3168 | *A-tag LoxP-HIS3-LoxP B-tag* |
| pEVE3169 | *A-tag LoxP-URA3-LoxP B-tag* |
| pEVE2176 | *B-tag* $P_{GPD1}$*-stuffer-*$T_{CYC1}$ *C-tag* |
| pEVE2177 | *C-tag* $P_{PGK1}$*-stuffer2-*$T_{ADH2}$ *D-tag* |
| pEVE2178 | *D-tag* $P_{TEF1}$*-stuffer3-*$T_{ENO2}$ *E-tag* |
| pEVE2179 | *E-tag* $P_{PDC1}$*-stuffer4-*$T_{FBA1}$ *F-tag* |
| pEVE2180 | *F-tag* $P_{TEF2}$*-stuffer-*$T_{PGI1}$ *G-tag* |
| pEVE2181 | *G-tag* $P_{PYK1}$*-stuffer-*$T_{ADH1}$ *H-tag* |
| pEVE22290 | *B-tag* $P_{PGK1}$*-stuffer-*$T_{CYC1}$ *D-tag* |
| pEVE1973 | *C-tag closing linker Z-tag* |
| pEVE1915 | *D-tag closing linker Z-tag* |
| pEVE1916 | *E-tag closing linker Z-tag* |
| pEVE1917 | *F-tag closing linker Z-tag* |
| pEVE1918 | *G-tag closing linker Z-tag* |
| pEVE1919 | *H-tag closing linker Z-tag* |
| pEVE2157 | *ARS/CEN/HIS3/*$P_{GPD1}$*-stuffer-*$T_{CYC1}$ |
| pEVE2159 | *ARS/CEN/LEU2/*$P_{PGK1}$*-stuffer2-*$T_{ADH2}$ |
| pEVE2358 | *ARS/CEN/URA3/* $P_{GPD1}$*-stuffer-*$T_{CYC1}$ |

**Table 8.** List of strains constructed in this study.

| Strain name | Genotype |
|---|---|
| BG | *MATα hoΔ0 his3Δ0 leu2Δ0 ura3Δ0 cat5Δ0::CAT5(I91M) mip1Δ0::MIP1(A661T) gal2Δ0::GAL2 sal1Δ0::SAL1* |
| DBR1 | BG *[ARS/CEN/URA3/*$P_{GPD1}$*-HaCHS-*$T_{CYC1}$*/*$P_{PGK1}$*-ScDFG10-*$T_{ADH2}$*/*$P_{TEF1}$*-At4CL2-*$T_{ENO2}$*/*$P_{PDC1}$*-AtPAL2-*$T_{FBA1}$*/*$P_{TEF2}$*-AmC4H-*$T_{PGI1}$*/*$P_{PYK1}$*-ScCPR1-*$T_{ADH1}$*]* |
| DBR2 | BG *[ARS/CEN/URA3/*$P_{GPD1}$*-HaCHS-*$T_{CYC1}$*/*$P_{PGK1}$*-ScTSC13-*$T_{ADH2}$*/*$P_{TEF1}$*-At4CL2-*$T_{ENO2}$*/*$P_{PDC1}$*-AtPAL2-*$T_{FBA1}$*/*$P_{TEF2}$*-AmC4H-*$T_{PGI1}$*/*$P_{PYK1}$*-ScCPR1-*$T_{ADH1}$*]* |
| DBR3 | BG *[ARS/CEN/URA3/*$P_{GPD1}$*-HaCHS-*$T_{CYC1}$*/*$P_{PGK1}$*-KlTSC13-*$T_{ADH2}$*/*$P_{TEF1}$*-At4CL2-*$T_{ENO2}$*/*$P_{PDC1}$*-AtPAL2-*$T_{FBA1}$*/*$P_{TEF2}$*-AmC4H-*$T_{PGI1}$*/*$P_{PYK1}$*-ScCPR1-*$T_{ADH1}$*]* |
| DBR4 | BG *[ARS/CEN/URA3/*$P_{GPD1}$*-HaCHS-*$T_{CYC1}$*/*$P_{PGK1}$*-AtECR-*$T_{ADH2}$*/*$P_{TEF1}$*-At4CL2-*$T_{ENO2}$*/*$P_{PDC1}$*-AtPAL2-*$T_{FBA1}$*/*$P_{TEF2}$*-AmC4H-*$T_{PGI1}$*/*$P_{PYK1}$*-ScCPR1-*$T_{ADH1}$*]* |
| DBR5 | BG *[ARS/CEN/URA3/*$P_{GPD1}$*-HaCHS-*$T_{CYC1}$*/*$P_{PGK1}$*-GhECR2-*$T_{ADH2}$*/*$P_{TEF1}$*-At4CL2-*$T_{ENO2}$*/*$P_{PDC1}$*-AtPAL2-*$T_{FBA1}$*/*$P_{TEF2}$*-AmC4H-*$T_{PGI1}$*/*$P_{PYK1}$*-ScCPR1-*$T_{ADH1}$*]* |
| DBR6 | BG *[ARS/CEN/URA3/*$P_{GPD1}$*-HaCHS-*$T_{CYC1}$*/*$P_{PGK1}$*-MdECR-*$T_{ADH2}$*/*$P_{TEF1}$*-At4CL2-*$T_{ENO2}$*/*$P_{PDC1}$*-AtPAL2-*$T_{FBA1}$*/*$P_{TEF2}$*-AmC4H-*$T_{PGI1}$*/*$P_{PYK1}$*-ScCPR1-*$T_{ADH1}$*]* |
| DBR7 | BG *[ARS/CEN/URA3/*$P_{GPD1}$*-HaCHS-*$T_{CYC1}$*/*$P_{PGK1}$*-MdENRL3-*$T_{ADH2}$*/*$P_{TEF1}$*-At4CL2-*$T_{ENO2}$*/*$P_{PDC1}$*-AtPAL2-*$T_{FBA1}$*/*$P_{TEF2}$*-AmC4H-*$T_{PGI1}$*/*$P_{PYK1}$*-ScCPR1-*$T_{ADH1}$*]* |
| DBR8 | BG *[ARS/CEN/URA3/*$P_{GPD1}$*-HaCHS-*$T_{CYC1}$*/*$P_{PGK1}$*-MdENRL5-*$T_{ADH2}$*/*$P_{TEF1}$*-At4CL2-*$T_{ENO2}$*/*$P_{PDC1}$*-AtPAL2-*$T_{FBA1}$*/*$P_{TEF2}$*-AmC4H-*$T_{PGI1}$*/*$P_{PYK1}$*-ScCPR1-*$T_{ADH1}$*]* |
| DBR9 | BG *[ARS/CEN/URA3/*$P_{GPD1}$*-HaCHS-*$T_{CYC1}$*/*$P_{PGK1}$*-MdHCDBR-*$T_{ADH2}$*/*$P_{TEF1}$*-At4CL2-*$T_{ENO2}$*/*$P_{PDC1}$*-AtPAL2-*$T_{FBA1}$*/*$P_{TEF2}$*-AmC4H-*$T_{PGI1}$*/*$P_{PYK1}$*-ScCPR1-*$T_{ADH1}$*]* |

| | |
|---|---|
| DBR10 | BG *[ARS/CEN/URA3/$P_{GPD1}$-HaCHS-$T_{CYC1}$/$P_{PGK1}$-ErERED-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| DBR11 | BG *[ARS/CEN/URA3/$P_{GPD1}$-HaCHS-$T_{CYC1}$/$P_{PGK1}$-RiZS1-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| DBR12 | BG *[ARS/CEN/URA3/$P_{GPD1}$-HaCHS-$T_{CYC1}$/$P_{PGK1}$-stuffer2-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| PAR1 | BG *[ARS/CEN/URA3/$P_{GPD1}$-HaCHS-$T_{CYC1}$/$P_{PGK1}$-ScTSC13-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| PAR2 | BG *[ARS/CEN/URA3/$P_{GPD1}$-HaCHS-$T_{CYC1}$/$P_{PGK1}$-stuffer2-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| PAR3 | BG *[ARS/CEN/URA3/$P_{GPD1}$-stuffer1-$T_{CYC1}$/$P_{PGK1}$-ScTSC13-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| PAR4 | BG *[ARS/CEN/URA3/$P_{GPD1}$-stuffer1-$T_{CYC1}$/$P_{PGK1}$-stuffer2-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| PAR5 | BG *[ARS/CEN/URA3/$P_{GPD1}$-stuffer1-$T_{CYC1}$/$P_{PGK1}$-ScTSC13-$T_{ADH2}$/$P_{TEF1}$-stuffer3-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| PAR6 | BG *[ARS/CEN/URA3/$P_{GPD1}$-stuffer1-$T_{CYC1}$/$P_{PGK1}$-stuffer2-$T_{ADH2}$/$P_{TEF1}$-stuffer3-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| PIN1 | BG *[ARS/CEN/URA3/$P_{GPD1}$-HaCHS-$T_{CYC1}$/$P_{PGK1}$-stuffer2-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$]* |
| PIN2 | BG *[ARS/CEN/URA3/$P_{GPD1}$-HaCHS-$T_{CYC1}$/$P_{PGK1}$-ScTSC13-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$]* |
| CHS1 | BG *[ARS/CEN/URA3/$P_{GPD1}$-HaCHS-$T_{CYC1}$/$P_{PGK1}$-ScTSC13-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| CHS2 | BG *[ARS/CEN/URA3/$P_{GPD1}$-PcCHS-$T_{CYC1}$/$P_{PGK1}$-ScTSC13-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| CHS3 | BG *[ARS/CEN/URA3/$P_{GPD1}$-PhCHS-$T_{CYC1}$/$P_{PGK1}$-ScTSC13-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| CHS4 | BG *[ARS/CEN/URA3/$P_{GPD1}$-HvCHS1-$T_{CYC1}$/$P_{PGK1}$-ScTSC13-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| CHS5 | BG *[ARS/CEN/URA3/$P_{GPD1}$-HvCHS2-$T_{CYC1}$/$P_{PGK1}$-ScTSC13-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| CHS6 | BG *[ARS/CEN/URA3/$P_{GPD1}$-SbCHS-$T_{CYC1}$/$P_{PGK1}$-ScTSC13-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| CHS7 | BG *[ARS/CEN/URA3/$P_{GPD1}$-MdCHS1-$T_{CYC1}$/$P_{PGK1}$-ScTSC13-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| CHS8 | BG *[ARS/CEN/URA3/$P_{GPD1}$-MdCHS2-$T_{CYC1}$/$P_{PGK1}$-ScTSC13-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| CHS9 | BG *[ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-ScTSC13-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$]* |
| PHZ1 | DBR2 *[ARS/CEN/LEU2/$P_{GPD1}$-MdUGT88F1-$T_{CYC1}$]* |
| PHZ2 | DBR2 *[ARS/CEN/LEU2/$P_{GPD1}$-MdUGT88A1-$T_{CYC1}$]* |
| PHZ3 | DBR2 *[ARS/CEN/LEU2/$P_{GPD1}$-PcUGT88F2-$T_{CYC1}$]* |
| PHZ4 | DBR2 *[ARS/CEN/LEU2/$P_{GPD1}$-DcGT4-$T_{CYC1}$]* |
| NOT1 | DBR2 *[ARS/CEN/LEU2/$P_{GPD1}$-OsCGT-$T_{CYC1}$]* |
| UGTn | DBR2 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| TRI1 | DBR2 *[ARS/CEN/LEU2/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-AtUGT73B2-$T_{ENO2}$]* |

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| TRI2 | DBR2 *[ARS/CEN/LEU2/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-AtUGT76D1-$T_{ENO2}$]* |
| TRI3 | DBR2 *[ARS/CEN/LEU2/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-AtUGT84B1-$T_{ENO2}$]* |
| NDC1 | DBR2 *[ARS/CEN/LEU2/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-AtUGT73B2-$T_{ENO2}$/$P_{PDC1}$-Cm1,2RhaT-$T_{FBA1}$/$P_{TEF2}$-AtRHM2-$T_{PGI1}$]* |
| HYP1 | DBR2 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-OsF3'H-$T_{ADH2}$/$P_{TEF1}$-AtATR1-$T_{ENO2}$]* |
| HYP2 | DBR2 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-PhF3'H-$T_{ADH2}$/$P_{TEF1}$-AtATR1-$T_{ENO2}$]* |
| HYP3 | DBR2 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-PfF3'H-$T_{ADH2}$/$P_{TEF1}$-AtATR1-$T_{ENO2}$]* |
| HYP4 | DBR2 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-AcF3'H-$T_{ADH2}$/$P_{TEF1}$-AtATR1-$T_{ENO2}$]* |
| HYP5 | DBR2 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-MdF3'H1-$T_{ADH2}$/$P_{TEF1}$-AtATR1-$T_{ENO2}$]* |
| HYP6 | DBR2 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-MdF3'H2-$T_{ADH2}$/$P_{TEF1}$-AtATR1-$T_{ENO2}$]* |
| HYP7 | DBR2 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-CsCH3H-$T_{ADH2}$/$P_{TEF1}$-AtATR1-$T_{ENO2}$]* |
| HYP8 | DBR2 *[ARS/CEN/LEU2/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-AtATR1-$T_{ENO2}$]* |
| NAR1 | *BG XI-3::loxP-URA3-loxP/$P_{GPD1}$-HaCHS-$T_{CYC1}$/$P_{PGK1}$-MsCHI-$T_{ADH2}$/$P_{TEF1}$-At4CL2-$T_{ENO2}$/$P_{PDC1}$-AtPAL2-$T_{FBA1}$/$P_{TEF2}$-AmC4H-$T_{PGI1}$/$P_{PYK1}$-ScCPR1-$T_{ADH1}$* |
| NAR2 | NAR1 *[ARS/CEN/LEU2/$P_{PGK1}$-stuffer-$T_{ADH2}$]* |
| ERI1 | NAR1 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-VvF3'H-$T_{ADH2}$/$P_{TEF1}$-AtCPR1-$T_{ENO2}$]* |
| ERI2 | NAR1 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-PhF3'H-$T_{ADH2}$/$P_{TEF1}$-AtCPR1-$T_{ENO2}$]* |
| ERI3 | NAR1 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-OsF3'H-$T_{ADH2}$/$P_{TEF1}$-AtCPR1-$T_{ENO2}$]* |
| PHF1 | NAR1 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-CrF3'5'H-$T_{ADH2}$/$P_{TEF1}$-AtCPR1-$T_{ENO2}$]* |
| PHF2 | NAR1 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-PhF3'5'H-$T_{ADH2}$/$P_{TEF1}$-AtCPR1-$T_{ENO2}$]* |
| PHF3 | NAR1 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-OhF3'5'H-$T_{ADH2}$/$P_{TEF1}$-AtCPR1-$T_{ENO2}$]* |
| PHF4 | NAR1 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-SlF3'5'H-$T_{ADH2}$/$P_{TEF1}$-AtCPR1-$T_{ENO2}$]* |
| PHF5 | NAR1 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-CiF3'5'H-$T_{ADH2}$/$P_{TEF1}$-AtCPR1-$T_{ENO2}$]* |
| PHF6 | NAR1 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-stuffer2-$T_{ADH2}$/$P_{TEF1}$-AtCPR1-$T_{ENO2}$]* |
| DHK1 | NAR1 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-FaF3H-$T_{ENO2}$]* |
| DHK2 | NAR1 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$]* |
| DHK3 | NAR1 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-NtF3H-$T_{ENO2}$]* |
| DHK4 | NAR1 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-stuffer3-$T_{ENO2}$]* |
| AFZ1 | NAR2 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AtDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| AFZ2 | NAR2 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| AFZ3 | NAR2 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| AFZ4 | NAR2 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| AFZ5 | NAR2 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-MdDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| AFZ6 | NAR2 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-stuffer4-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| CAT1 | ERI2 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AtDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| CAT2 | ERI2 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |

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| CAT3 | ERI2 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| CAT4 | ERI2 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| CAT5 | ERI2 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-MdDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| CAT6 | ERI2 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-stuffer4-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| GAL1 | PHF4 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AtDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| GAL2 | PHF4 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| GAL3 | PHF4 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| GAL4 | PHF4 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| GAL5 | PHF4 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-MdDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| GAL6 | PHF4 *[ARS/CEN/HIS3/$P_{PGK1}$-stuffer-$T_{CYC1}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-stuffer4-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| PEL1 | NAR1 *XI-2::loxP-HIS5-loxP/$P_{GPD1}$-PhANS-$T_{CYC1}$/$P_{PGK1}$-stuffer-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$* |
| CYA1 | NAR1 *XI-2::loxP-HIS5-loxP/$P_{GPD1}$-PhANS-$T_{CYC1}$/$P_{PGK1}$-PhF3'H-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$/$P_{TEF2}$-stuffer-$T_{PGI1}$/$P_{PYK1}$-AtCPR1-$T_{ADH1}$* |
| DEL1 | NAR1 *XI-2::loxP-HIS5-loxP/$P_{GPD1}$-PhANS-$T_{CYC1}$/$P_{PGK1}$-SlF3'5'H-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$/$P_{TEF2}$-stuffer-$T_{PGI1}$/$P_{PYK1}$-AtCPR1-$T_{ADH1}$* |
| PGT1 | PEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-OsA3GT-$T_{CYC1}$]* |
| PGT2 | PEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-GtA3GT-$T_{CYC1}$]* |
| PGT3 | PEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-PhA3GT-$T_{CYC1}$]* |
| PGT4 | PEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-AtA3GT-$T_{CYC1}$]* |
| PGT5 | PEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT1-$T_{CYC1}$]* |
| PGT6 | PEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-VvA3GT-$T_{CYC1}$]* |
| PGT7 | PEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$]* |
| PGT8 | PEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$]* |
| PGT9 | PEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-GtA3GT-$T_{CYC1}$]* |
| PGT10 | PEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGT1 | CYA1 *[ARS/CEN/LEU2/$P_{GPD1}$-OsA3GT-$T_{CYC1}$]* |
| CGT2 | CYA1 *[ARS/CEN/LEU2/$P_{GPD1}$-GtA3GT-$T_{CYC1}$]* |
| CGT3 | CYA1 *[ARS/CEN/LEU2/$P_{GPD1}$-PhA3GT-$T_{CYC1}$]* |
| CGT4 | CYA1 *[ARS/CEN/LEU2/$P_{GPD1}$-AtA3GT-$T_{CYC1}$]* |
| CGT5 | CYA1 *[ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT1-$T_{CYC1}$]* |
| CGT6 | CYA1 *[ARS/CEN/LEU2/$P_{GPD1}$-VvA3GT-$T_{CYC1}$]* |
| CGT7 | CYA1 *[ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$]* |
| CGT8 | CYA1 *[ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$]* |
| CGT9 | CYA1 *[ARS/CEN/LEU2/$P_{GPD1}$-GtA3GT-$T_{CYC1}$]* |

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| CGT10 | CYA1 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGT1 | DEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-OsA3GT-$T_{CYC1}$]* |
| DGT2 | DEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-GtA3GT-$T_{CYC1}$]* |
| DGT3 | DEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-PhA3GT-$T_{CYC1}$]* |
| DGT4 | DEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-AtA3GT-$T_{CYC1}$]* |
| DGT5 | DEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT1-$T_{CYC1}$]* |
| DGT6 | DEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-VvA3GT-$T_{CYC1}$]* |
| DGT7 | DEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$]* |
| DGT8 | DEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$]* |
| DGT9 | DEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-GtA3GT-$T_{CYC1}$]* |
| DGT10 | DEL1 *[ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PANS1 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-InANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$]* |
| PANS2 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-GhANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$]* |
| PANS3 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-MdANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$]* |
| PANS4 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$]* |
| PANS5 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-FaANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$]* |
| PANS6 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-PcANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$]* |
| PANS7 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-IbANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$]* |
| PANS8 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-StANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$]* |
| PANS9 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-MsANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$]* |
| PANS10 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-GbANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$]* |
| PANS11 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-ZmANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$]* |
| PANS12 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-AtANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$]* |
| PANS13 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-OsANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$]* |
| PANS14 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-AcANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$]* |
| PANS15 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$]* |
| CANS1 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-InANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$]* |
| CANS2 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-GhANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$]* |

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| CANS3 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-MdANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$]* |
| CANS4 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$]* |
| CANS5 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-FaANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$]* |
| CANS6 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-PcANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$]* |
| CANS7 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-IbANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$]* |
| CANS8 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-StANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$]* |
| CANS9 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-MsANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$]* |
| CANS10 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-GbANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$]* |
| CANS11 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-ZmANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$]* |
| CANS12 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-AtANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$]* |
| CANS13 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-OsANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$]* |
| CANS14 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-AcANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$]* |
| CANS15 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$]* |
| DANS1 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-InANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$]* |
| DANS2 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-GhANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$]* |
| DANS3 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-MdANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$]* |
| DANS4 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-PhANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$]* |
| DANS5 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-FaANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$]* |
| DANS6 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-PcANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$]* |
| DANS7 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-IbANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$]* |
| DANS8 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-StANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$]* |
| DANS9 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-MsANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$]* |
| DANS10 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-GbANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$]* |
| DANS11 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-ZmANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$]* |

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| DANS12 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-AtANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$]* |
| DANS13 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-OsANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$]* |
| DANS14 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-AcANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$]* |
| DANS15 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$]* |
| PDFR1 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-PcANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| PDFR2 | NAR2 *[ARS/CEN/HIS3/$P_{GPD1}$-PcANS-$T_{CYC1}$/$P_{PGK1}$-DcA3GT-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-stuffer4-$T_{FBA1}$]* |
| CDFR1 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-MdANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| CDFR2 | ERI2 *[ARS/CEN/HIS3/$P_{GPD1}$-MdANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-stuffer4-$T_{FBA1}$]* |
| DDFR1 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-PcANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$/$P_{TEF2}$-VvLAR-$T_{PGI1}$]* |
| DDFR2 | PHF4 *[ARS/CEN/HIS3/$P_{GPD1}$-PcANS-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-stuffer4-$T_{FBA1}$]* |
| PEL2 | NAR1 *loxP-ura3Δ0-loxP::loxP XI-2::loxP/$P_{GPD1}$-PhANS-$T_{CYC1}$/$P_{PGK1}$-stuffer-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-AaDFR-$T_{FBA1}$* |
| CYA2 | NAR1 *loxP-ura3Δ0-loxP::loxP XI-2::loxP/$P_{GPD1}$-PhANS-$T_{CYC1}$/$P_{PGK1}$-PhF3'H-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-PtDFR-$T_{FBA1}$/$P_{TEF2}$-stuffer-$T_{PGI1}$/$P_{PYK1}$-AtCPR1-$T_{ADH1}$* |
| DEL2 | NAR1 *loxP-ura3Δ0-loxP::loxP XI-2::loxP/$P_{GPD1}$-PhANS-$T_{CYC1}$/$P_{PGK1}$-SlF3'5'H-$T_{ADH2}$/$P_{TEF1}$-MdF3H-$T_{ENO2}$/$P_{PDC1}$-IhDFR-$T_{FBA1}$/$P_{TEF2}$-stuffer-$T_{PGI1}$/$P_{PYK1}$-AtCPR1-$T_{ADH1}$* |
| PGST1 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-ZmBZ2-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PGST2 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PGST3 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-AtTT19-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PGST4 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-AtTT19-3-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PGST5 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-AtTT19-4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PGST6 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST1-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PGST7 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST2-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PGST8 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST3-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PGST9 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PGST10 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST5-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PGST11 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PfGST1-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |

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| PGST12 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-CsGST3-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PGST13 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-TaGSTL1-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PGST14 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-stuffer-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-DcA3GT-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PGST15 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PGST16 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-stuffer-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST1 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-ZmBZ2-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST2 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST3 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-AtTT19-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST4 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-AtTT19-3-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST5 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-AtTT19-4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST6 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST1-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST7 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST2-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST8 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST3-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST9 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST10 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST5-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST11 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-PfGST1-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST12 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-CsGST3-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST13 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-TaGSTL1-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST14 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-stuffer-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST15 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CGST16 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-stuffer-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGST1 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-ZmBZ2-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGST2 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGST3 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-AtTT19-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |

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| DGST4 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-AtTT19-3-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGST5 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-AtTT19-4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGST6 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST1-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGST7 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST2-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGST8 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST3-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGST9 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGST10 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST5-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGST11 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PfGST1-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGST12 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-CsGST3-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGST13 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-TaGSTL1-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGST14 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-stuffer-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGST15 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| DGST16 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-stuffer-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-stuffer-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| CTR1 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-AtTT12-$T_{CYC1}$]* |
| CTR2 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-SlMTP77-$T_{CYC1}$]* |
| CTR3 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-VvAM1-$T_{CYC1}$]* |
| CTR4 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-VvAM3-$T_{CYC1}$]* |
| CTR5 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-MtMATE1-$T_{CYC1}$]* |
| CTR6 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-BnTT12-1-$T_{CYC1}$]* |
| CTR7 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-MdMATE1-$T_{CYC1}$]* |
| CTR8 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-MdMATE2-$T_{CYC1}$]* |
| CTR9 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-VvMATE1-$T_{CYC1}$]* |
| CTR10 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-VvMATE2-$T_{CYC1}$]* |
| CTR11 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-GaTT12a-$T_{CYC1}$]* |

| | |
|---|---|
| CTR12 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-ZmMRP3-$T_{CYC1}$]* |
| CTR13 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-VvABCC1-$T_{CYC1}$]* |
| CTR14 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-VvGST4-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$]* |
| PMOD1 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$]* |
| PMOD2 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-Dv3MAT-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$]* |
| PMOD3 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-FaA3GT-$T_{ADH2}$/$P_{TEF1}$-stuffer3-$T_{ENO2}$/$P_{PDC1}$-stuffer4-$T_{FBA1}$/$P_{TEF2}$-VaA5GT-$T_{PGI1}$]* |
| PMOD4 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-FaA3GT-$T_{ADH2}$/$P_{TEF1}$-CtA3p5pGT-$T_{ENO2}$]* |
| PMOD5 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-Dv3MAT-$T_{CYC1}$/$P_{PGK1}$-FaA3GT-$T_{ADH2}$/$P_{TEF1}$-CtA3p5pGT-$T_{ENO2}$]* |
| PMOD6 | PEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-FaA3GT-$T_{ADH2}$/$P_{TEF1}$-stuffer3-$T_{ENO2}$/$P_{PDC1}$-NsA3GRhaT-$T_{FBA1}$/$P_{TEF2}$-AtRHM2-$T_{PGI1}$]* |
| CMOD1 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$]* |
| CMOD2 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-Dv3MAT-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$]* |
| CMOD3 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-FaA3GT-$T_{ADH2}$/$P_{TEF1}$-stuffer3-$T_{ENO2}$/$P_{PDC1}$-stuffer4-$T_{FBA1}$/$P_{TEF2}$-VaA5GT-$T_{PGI1}$]* |
| CMOD4 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-FaA3GT-$T_{ADH2}$/$P_{TEF1}$-CtA3p5pGT-$T_{ENO2}$]* |
| CMOD5 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-Dv3MAT-$T_{CYC1}$/$P_{PGK1}$-FaA3GT-$T_{ADH2}$/$P_{TEF1}$-CtA3p5pGT-$T_{ENO2}$]* |
| CMOD6 | CYA2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-FaA3GT-$T_{ADH2}$/$P_{TEF1}$-stuffer3-$T_{ENO2}$/$P_{PDC1}$-NsA3GRhaT-$T_{FBA1}$/$P_{TEF2}$-AtRHM2-$T_{PGI1}$]* |
| DMOD1 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$]* |
| DMOD2 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-Dv3MAT-$T_{CYC1}$/$P_{PGK1}$-FaA3GT2-$T_{ADH2}$]* |
| DMOD3 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-FaA3GT-$T_{ADH2}$/$P_{TEF1}$-stuffer3-$T_{ENO2}$/$P_{PDC1}$-stuffer4-$T_{FBA1}$/$P_{TEF2}$-VaA5GT-$T_{PGI1}$]* |
| DMOD4 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-FaA3GT-$T_{ADH2}$/$P_{TEF1}$-CtA3p5pGT-$T_{ENO2}$]* |
| DMOD5 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-Dv3MAT-$T_{CYC1}$/$P_{PGK1}$-FaA3GT-$T_{ADH2}$/$P_{TEF1}$-CtA3p5pGT-$T_{ENO2}$]* |
| DMOD6 | DEL2 *[ARS/CEN/HIS3/$P_{GPD1}$-PhAN9-$T_{CYC1}$] [ARS/CEN/LEU2/$P_{GPD1}$-FaA3GT2-$T_{CYC1}$] [ARS/CEN/URA3/$P_{GPD1}$-stuffer-$T_{CYC1}$/$P_{PGK1}$-FaA3GT-$T_{ADH2}$/$P_{TEF1}$-stuffer3-$T_{ENO2}$/$P_{PDC1}$-NsA3GRhaT-$T_{FBA1}$/$P_{TEF2}$-AtRHM2-$T_{PGI1}$]* |

## 4.5 Yeast growth and metabolite extraction

For compound analysis, cultures of yeast strains were prepared in 96 half-deepwell microplates (DWP) with the System Duetz (EnzyScreen, Heemstede, Netherlands) at 30°C, 5 cm shaking diameter, and 300 RPM. Precultures were grown for 24 hours from single colonies in 300 µl SC dropout medium, as required for selection of plasmids. Batch main cultures were inoculated in 300 µl of the same medium to a 1:100 dilution of the preculture and grown for 72 hours. Fed-batch main cultures were inoculated in 300 µl of Feed in Time™ medium to an OD of 0.3 and grown for 120 hours. For analysis of supernatants and pellets, cells were separated from the culture broth by centrifugation at 4000 g for 5 minutes. Then cell pellets were resuspended in the same volume of $ddH_2O$ as used in the culture. For extraction and stabilization of compounds, 150 µl culture broth, supernatant, or resuspended pellet was mixed with 150 µl of methanol (for dihydrochalcones in section 5.1) or acidified methanol (1% hydrochloric acid) (for anthocyanins in section 5.2 and 5.3) and incubated for 10 minutes in a 96 DWP at 30°C, 5 cm shaking diameter, and 300 RPM and subsequently clarified by centrifugation at 4000 g for 5 minutes.

## 4.6 Quantification of compounds by UPLC-MS

Two different setups of UPLC-MS, for analyzing dihydrochalcones (section 5.1) and anthocyanins (sections 5.2 and 5.3) respectively, were used.

For quantification of dihydrochalcones, clarified broth extracts were diluted four times with 50% methanol and 2 µl was injected on a Waters Acquity ultra performance liquid chromatography system coupled to a Waters Acquity triple quadrupole mass detector (Milford, Massachusetts, USA). Separation of the compounds was achieved on a Waters Acquity UPLC® BEH C18 column (1.7 µm, 2.1 mm x 50 mm) kept at 55°C. Mobile phases A and B were water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid, respectively. A flow of 0.6 ml/min was used. The gradient profile was as follow: 0.3 min constant at 10% B, a linear gradient from 10% B to 25% B in 3.7 min, a second linear gradient from 25% B to 100% B in 1 min, a wash for 1 min at 100% B, and back to the initial condition of 10% B for 0.6 min. The mass analyzer was equipped with an electrospray source and operated in negative mode. Capillary voltage was 3.0 kV; the source was kept at 150°C and the desolvation

temperature was 350°C. Desolvation and cone gas flow were 500 l/h and 50 l/h, respectively. $[M-H]^-$ ions of compounds of interest were tracked in SIR mode. Compounds were quantified using a quadratic calibration curve with authentic standards ranging from 0.156 mg/l to 80 mg/l for *p*-coumaric acid and phloretic acid or from 0.039 mg/l to 20 mg/l for all other compounds.

For quantification of metabolites of the anthocyanin pathway, a calibration curve with authentic standards ranging from 0.02 mg/l to 4 mg/l was injected along with clarified broth extracts. Generally, extracts were diluted with 50% methanol, 0.5% hydrochloric acid to be within the range of the calibration curve. 1 µl was injected on a Waters Acquity ultra performance liquid chromatography system coupled to a Waters Xevo G2 XS Tof mass detector (Milford, Massachusetts, USA). Separation of compounds was achieved with the same column, temperature, solvents, and gradient profile as for dihydrochalcones. The mass analyzer was equipped with an electrospray source and operated in positive mode for quantification of anthocyanins in section 5.2 and in negative mode for all other compounds and anthocyanins in section 5.3. Capillary voltage was 2.0 kV and 1.0 kV for positive and negative mode, respectively; the source was kept at 150°C and the desolvation temperature was 500°C. Desolvation and cone gas flow were 1000 l/h and 150 l/h, respectively. For each compound of interest we calculated peak areas of the extracted ion chromatograms of the respective $[M-H]^-$, $[M+H]^+$, or $[M]^+$ ions using a mass window of 0.02 Da, and quantified using a linear calibration curve of authentic standards.

# 5 Results and Discussion

## 5.1 Metabolic engineering of *S. cerevisiae* for *de novo* production of dihydrochalcones with known antioxidant, antidiabetic, and sweet tasting properties

### 5.1.1 Testing DBRs for production of phloretin in metabolically engineered yeast

Several previous studies focusing on heterologous production of flavonoids or stilbenoids in *S. cerevisiae* reported the appearance of phloretic acid or phloretin as by-products and attributed this to an unspecific activity of some native yeast DBR acting on *p*-coumaric acid or *p*-coumaroyl-CoA to form phloretic acid or *p*-dihydrocoumaroyl-CoA, respectively (Beekwilder et al., 2006; Jiang, Wood, & Morgan, 2005; Koopman et al., 2012; Luque et al., 2014). By screening of 25 homozygous and heterozygous deletion mutants of *S. cerevisiae* for genes that might be involved in this DBR shunt activity, *ScDFG10* and *ScTSC13* were identified as possible candidates (Lehka et al., 2017). ScDfg10 is a polyprenol reductase involved in the biosynthesis of dolichol, the precursor for protein N-glycosylation (Cantagrel et al., 2010). ScTsc13 is a very long chain (VLC) enoyl-CoA reductase, catalyzing the double bond reduction in each cycle of VLC fatty acid elongation (Kohlwein et al., 2001). These two enzymes were chosen for overexpression in the attempt to construct a pathway to phloretin in *S. cerevisiae*. In addition to the DBRs, the phloretin pathway comprised the genes *AtPAL2*, *AmC4H*, *ScCPR1*, *At4CL2*, and *HaCHS*, which were previously found to encode a functional naringenin pathway in yeast (data not shown). Despite the absence of a chalcone isomerase (CHI), naringenin production was expected in these strains, since the isomerization of naringenin chalcone to naringenin is known to happen spontaneously (Mol, Robbins, Dixon, & Veltkamp, 1985). This spontaneous isomerization was also observed for authentic standards of naringenin chalcone, so naringenin was used for indirect quantification of CHS products derived from *p*-coumaroyl-CoA. Preliminary data suggested that overexpression of *ScTSC13* resulted in increased phloretin production without affecting final cell density (Figure 6A). Moreover, essentially no effect was observed on the long chain and VLC free fatty acid profile (Figure 6B and Figure 6C). This suggested that overexpression of *ScTSC13*

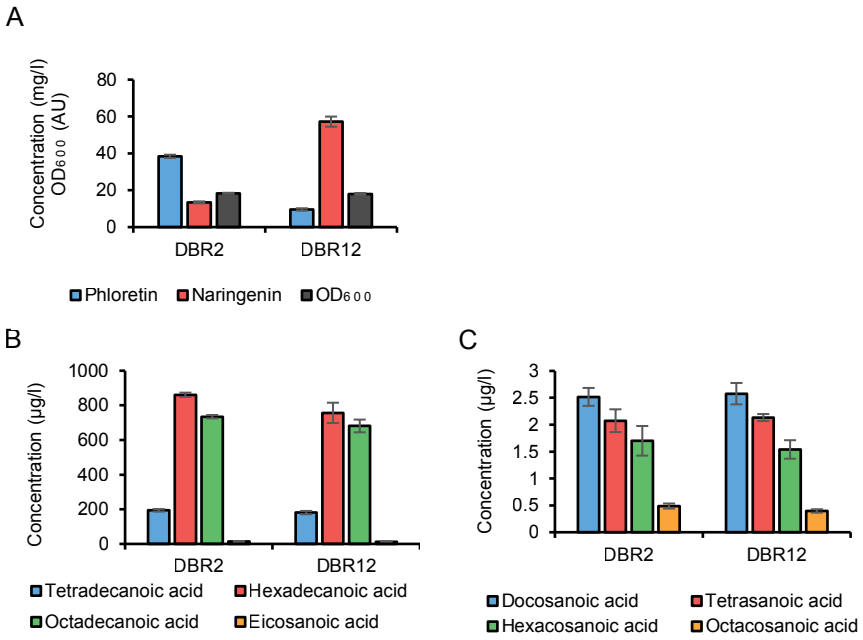


**Figure 6.** Strains DBR2 and DBR12, expressing a phloretin pathway with *AtPAL2*, *AmC4H*, *ScCPR1*, *At4CL2*, and *HaCHS* together with *ScTSC13* (DBR2) or no DBR (DBR12) on an HRT plasmid, were compared for production of phloretin, naringenin, and final optical density at 600nm (A), for production of long chain free fatty acids (B), and for production of very long chain free fatty acids (C).

alone has no impact on the activity of the four-enzyme VLC fatty acid elongation machinery, which additionally comprises Sc*ELO2* or Sc*ELO3*, Sc*IFA38*, and Sc*PHS1* (Dickson, 2008). Obviously, the DBR activity in the phloretin pathway is not the native function of ScTsc13 and we wondered to what extent this was a unique feature of this enzyme. We therefore tested four homologous enzymes for this activity: KlTsc13 from *K. lactis* was chosen as an example of a functionally similar enzyme from another yeast. AtEcr from *A. thaliana* and GhEcr2 from cotton were selected as they have previously been shown to complement *S. cerevisiae* lacking ScTsc13 activity (Gable, Garton, Napier, & Dunn, 2004; Song et al., 2009), thus confirming that they can be functionally expressed in yeast. Additionally, the *M.* x *domestica* MdEcr homologue was selected due to the high dihydrochalcone content of this plant. On top of that, several recently identified DBRs were tested for activity in our synthetic phloretin pathway: three enzymes from apple were previously shown to be implicated in dihydrochalcone production, either by RNAi experiments (MdEnrl3) or by *in vitro* reaction studies (MdEnrl5, MdHcdbr) (Dare, Tomes, Cooney, et al., 2013; Ibdah et al., 2014). ErEred was selected because it has been shown to produce phloretin from naringenin chalcone when *ErERED* is expressed in anaerobically grown *E. coli* (Gall et al., 2014). Finally, RiZs1 from raspberry was chosen because it reduces the double bond of 4-

hydroxybenzalacetone, which is structurally very similar to *p*-coumaric acid (Koeduka et al., 2011). Twelve HRT plasmids were constructed, co-expressing *AtPAL2*, *AmC4H*, *ScCPR1*, *At4CL2*, and *HaCHS* together with either one of the eleven DBRs or a negative control without DBR, to make the strains DBR1 to DBR12, and production of naringenin and phloretin was quantified (Figure 7).

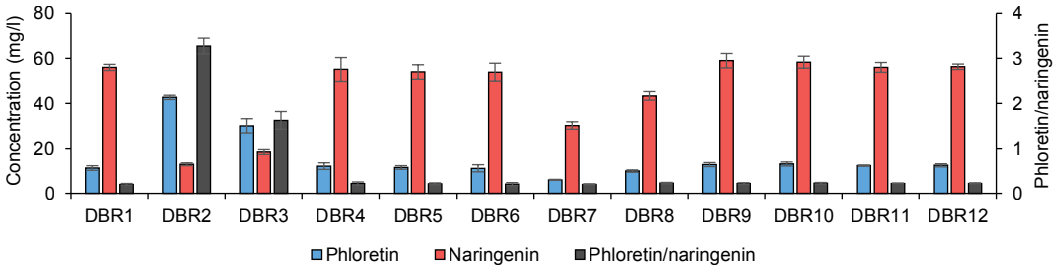


**Figure 7.** Production of phloretin and naringenin and the ratio of produced phloretin to naringenin by strains DBR1 to DBR12, expressing a phloretin pathway with *AtPAL2*, *AmC4H*, *ScCPR1*, *At4CL2*, *HaCHS,* and different DBRs (DBR1: *ScDFG10*; DBR2: *ScTSC13*; DBR3: *KlTSC13*; DBR4: *AtECR*; DBR5: *GhECR2*; DBR6: *MdECR*; DBR7: *MdENRL3*; DBR8: *MdENRL5*; DBR9: *MdHCDBR*; DBR10: *ErERED*: DBR11: *RiZS1*; DBR12: no DBR) on an HRT plasmid. Represented are average and standard deviation of three independent cultures.

Of the DBRs tested, only ScTsc13 and its close homologue KlTsc13 had any impact on the ratio of phloretin to naringenin. Strain DBR2, overexpressing *ScTSC13*, was most efficient in producing phloretin, reaching a final titer of 42.7 ± 0.9 mg/l and only 13.0 ± 0.6 mg/l of naringenin. As mentioned above, *ScTSC13* is an essential gene in *S. cerevisiae* and the corresponding enzyme catalyzes the double bond reduction of VLC trans-2-enoyl-CoA (Kohlwein et al., 2001). Like the native substrates of this enzyme, *p*-coumaroyl-CoA comprises an α,β-unsaturated CoA thioester functional group. Therefore, it seems likely that *p*-coumaroyl-CoA is the actual substrate of the ScTsc13 side activity.

### 5.1.2 Characterization of the phloretin pathway *in vivo*

The overexpression of *ScTSC13*, together with a heterologous pathway to naringenin chalcone, resulted in production of dihydrochalcones instead of flavonoids. In order to further elucidate the exact substrate of ScTsc13 in this pathway, and to identify potential bottlenecks, six truncated pathways were assembled on HRT plasmids to make strains PAR1-PAR6 (Table 9). These strains were tested for production of cinnamic acid, *p*-coumaric acid, phloretic acid, naringenin, and phloretin (Figure 8).

When the pathway was interrupted at the stage of *p*-coumaric acid by leaving out *At4CL2* and *HaCHS* (strains PAR5 and PAR6) only *p*-coumaric acid was produced. No phloretic or cinnamic acid was detected despite the overexpression of *ScTSC13*. This suggested that AmC4h was not limiting in the pathway and that *p*-coumaric acid was not a substrate of ScTsc13. When the pathway was extended to include *At4CL2* (strains PAR3 and PAR4) the yeast produced only phloretic acid, whereas the *p*-coumaric acid concentration was below the detection limit. This suggested that At4cl2 efficiently activated *p*-coumaric acid to *p*-coumaroyl-CoA, which was then reduced to *p*-dihydrocoumaroyl-CoA, even without overexpression of *ScTSC13*. *p*-Dihydrocoumaroyl-CoA was subsequently hydrolyzed to phloretic acid, by an unspecific enzymatic reaction or spontaneously. These results also showed that At4cl2 and ScTsc13 were not limiting under these experimental conditions.

**Table 9.** Genes on HRT plasmids in strains PAR1 to PAR6.

| Strain | Genes on corresponding HRT plasmid |
|---|---|
| PAR1 | AtPAL2, AmC4H, ScCPR1, At4CL2, HaCHS, ScTSC13 |
| PAR2 | AtPAL2, AmC4H, ScCPR1, At4CL2, HaCHS |
| PAR3 | AtPAL2, AmC4H, ScCPR1, At4CL2, ScTSC13 |
| PAR4 | AtPAL2, AmC4H, ScCPR1, At4CL2 |
| PAR5 | AtPAL2, AmC4H, ScCPR1, ScTSC13 |
| PAR6 | AtPAL2, AmC4H, ScCPR1 |

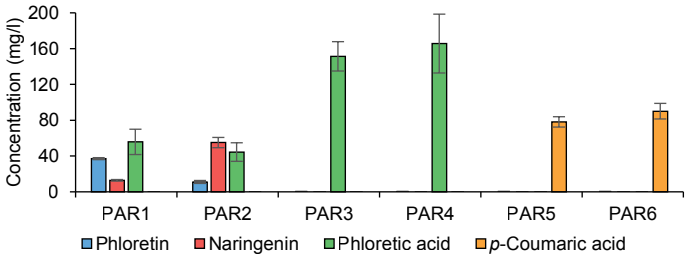


**Figure 8.** Production of phloretin, naringenin, phloretic acid, and *p*-coumaric acid by strains PAR1 to PAR6, containing an HRT plasmid with partial and full phloretin pathways as shown in Table 9. Represented are average and standard deviation of three independent cultures.

Strains PAR1 and PAR2, comprising the full-length pathway to naringenin chalcone, both produced phloretin and naringenin. The accumulation of both compounds most likely reflected the competition between ScTsc13 and HaChs for *p*-coumaroyl-CoA. As expected, the overexpression of *ScTSC13* (PAR1) clearly shifted the balance of production towards more phloretin and less naringenin compared to PAR2. Both strains accumulated phloretic acid, suggesting slow conversion of *p*-dihydrocoumaroyl-CoA to phloretin by HaChs, which seemed to present a bottleneck in

the current pathway. To confirm the gradual build-up of compounds measured after 72 hours, we performed a time course experiment using PAR1, analyzing the accumulation of relevant compounds (Figure 9). After a minor transient accumulation of *p*-coumaric acid during the initial exponential growth phase on glucose, there was a steady build-up of phloretic acid, as well as phloretin and naringenin, corresponding to a presumed slow reaction of HaChs.

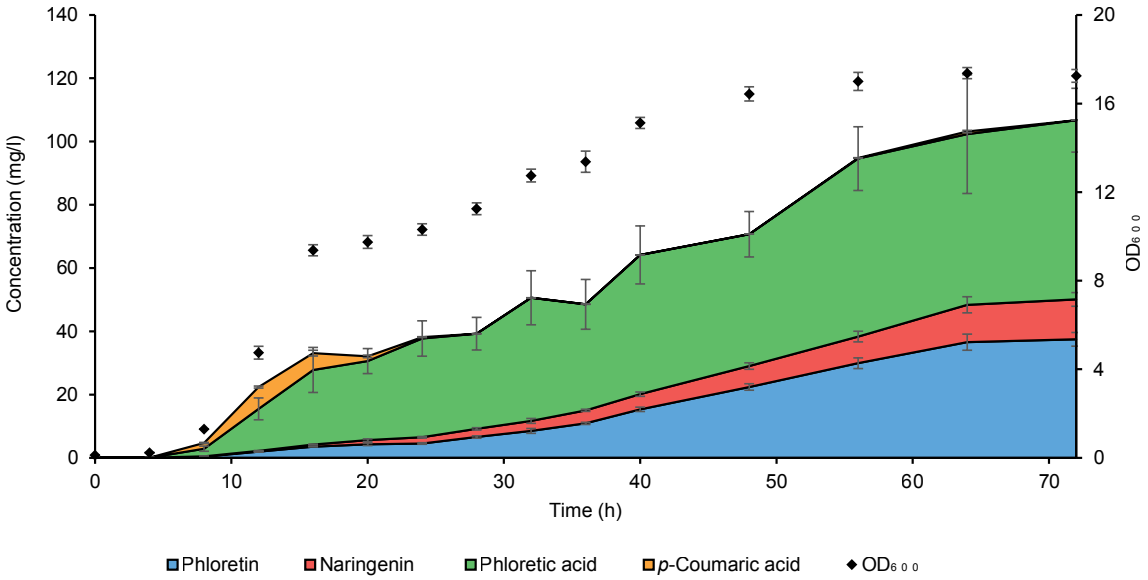


**Figure 9.** Time course of production of phloretin, naringenin, phloretic acid, and *p*-coumaric acid by strain PAR1 and corresponding optical density at 600 nm. Represented are average and standard deviation of four independent cultures.

These results support the hypothesis that *p*-coumaroyl-CoA is a substrate of ScTsc13 although we cannot completely exclude the possibility that the enzyme has activity on naringenin chalcone. However, this seems very unlikely, since naringenin chalcone does not share any structural similarity, nor the CoA functional group, with the natural substrates of the enzyme (Kohlwein et al., 2001). Furthermore, naringenin chalcone quickly isomerizes to naringenin, which does not contain the targeted double bond, and therefore is not a substrate of ScTsc13. In fact, in the current pathway naringenin is an unwanted by-product and, hence, a CHS that does not accept *p*-coumaroyl CoA would be highly desirable. This would greatly simplify balancing of the pathway, during further boosting and optimization, as there would be no competition with ScTsc13 for the same substrate. While HaChs represents the major limiting step to be alleviated in the heterologous pathway, another way to further increase phloretin production would be to increase the flux into the pathway, e.g. by boosting

phenylalanine production and increasing PAL activity. This approach successfully increased production of naringenin (Koopman et al., 2012), *p*-coumaric acid (Rodriguez, Kildegaard, Li, Borodina, & Nielsen, 2015), and resveratrol (M. Li et al., 2015) in previous studies, which all share phenylalanine as precursor.

### 5.1.3 Using *ScTSC13* for production of pinocembrin dihydrochalcone

While most dihydrochalcones found in nature are derived from phloretin, some plants, such as *Uvaria angolensis* (Hufford & Oguntimein, 1980) or *Mitrella kentia* (Benosman et al., 1997), accumulate also dihydrochalcone structures lacking the 4-hydroxy group. This means they are most likely derived from pinocembrin dihydrochalcone and are produced by CHS from dihydrocinnamoyl-CoA and three units of malonyl-CoA. Hence, the biosynthesis of these compounds would require a DBR acting on cinnamoyl-CoA to form dihydrocinnamoyl-CoA (Figure 10A). In order to test whether ScTsc13 can also catalyze this reaction, two HRT plasmids were constructed, creating strains PIN1 and PIN2, comprising the pathway to pinocembrin chalcone (*AtPAL2*, *At4CL2*, and *HaCHS*) either with or without additional overexpression of *ScTSC13*.

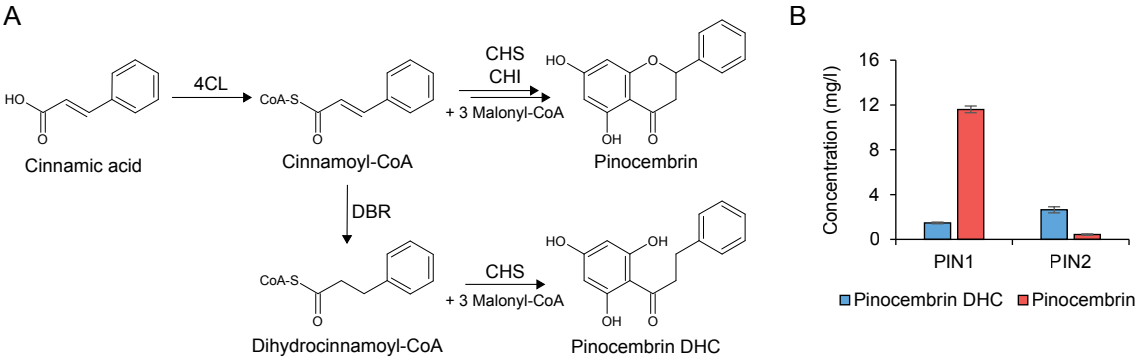


**Figure 10.** (A) Biosynthetic route from cinnamic acid to pinocembrin and proposed route to pinocembrin DHC. (B) Production of pinocembrin DHC and pinocembrin by strains PIN1 and PIN2, expressing a pathway to pinocembrin chalcone with *AtPAL2*, *At4CL2*, and *HaCHS* either without (PIN1) or with (PIN2) overexpression of *ScTSC13*, respectively. Represented are average and standard deviation of three independent cultures. CHS, chalcone synthase; DBR, double bond reductase; PAL, phenylalanine ammonia lyase; 4CL, 4-coumarate-CoA ligase.

These strains were cultured and the production of pinocembrin and pinocembrin dihydrochalcone was analyzed (Figure 10B). Strain PIN1, containing only the native expression of *ScTSC13*, produced 1.47 ± 0.07 mg/l of pinocembrin dihydrochalcone in addition to 11.6 ± 0.3 mg/l of pinocembrin. Overexpression of *ScTSC13* in strain PIN2

increased the pinocembrin dihydrochalcone production to 2.6 ± 0.3 mg/l and decreased the pinocembrin production to 0.43 ± 0.06 mg/l, demonstrating that ScTsc13 also accepts cinnamoyl-CoA. Generally, production of pinocembrin and pinocembrin dihydrochalcone in strain PIN2 was lower than production of naringenin and phloretin in strain DBR2, which might suggest that At4cl2 and HaChs prefer the hydroxylated substrates, or that the compounds lacking the 4-hydroxy group are more prone to degradation in yeast.

### 5.1.4 Testing CHS from various plant species for more specific phloretin production

The production of phloretin in strain DBR2 was unfortunately not very specific with naringenin accumulating as a major by-product to almost a third of the amount of phloretin produced (Figure 7). In an attempt to decrease this by-product formation, we tested a collection of eight different CHS enzymes from various plants for their ability to produce naringenin or phloretin in yeast. Two of the CHS genes were amplified from the peel of *M.* x *domestica* var. Golden Delicious, as this plant is known to accumulate high concentrations of dihydrochalcones (Dare, Tomes, Jones, et al., 2013; Gosch et al., 2009).

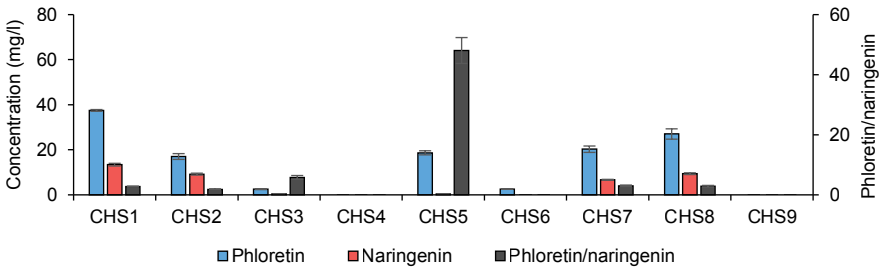


**Figure 11.** Production of phloretin and naringenin and the ratio of produced phloretin to naringenin by strains CHS1 to CHS9, expressing a phloretin pathway with *AtPAL2*, *AmC4H*, *ScCPR1*, *At4CL2*, and *ScTSC13* with different CHS (CHS1: *HaCHS*; CHS2: *PcCHS*; CHS3: *PhCHS*; CHS4: *HvCHS1*; CHS5: *HvCHS2*, CHS6: *SbCHS*; CHS7: *MdCHS1*; CHS8: *MdCHS2*; CHS9: no CHS) on an HRT plasmid. Represented are average and standard deviation of three independent cultures.

Nine HRT plasmids containing the pathway to *p*-dihydrocoumaroyl-CoA (*AtPAL2*, *AmC4H*, *ScCPR1*, *At4CL2*, *ScTSC13*) together with one of the eight CHS, or no CHS as negative control, were constructed to make strains CHS1 to CHS9 and the production of naringenin and phloretin was quantified (Figure 11). HaChs, which was originally chosen for the highest efficiency in producing naringenin in yeast (data not shown), was

found to be the most efficient CHS for phloretin production as well. However, most specific for phloretin production was strain CHS5 expressing *HvCHS2*. The phloretin/naringenin ratio of this strain exceeded the ratio of strain CHS1 expressing *HaCHS* by 17.2 ± 1.7 fold.

This result was unexpected because barley has not been reported to produce dihydrochalcones. In the original publication describing HvChs2, an unusual substrate preference for feruloyl-CoA and caffeoyl-CoA over *p*-coumaroyl-CoA and cinnamoyl-CoA was reported, while *p*-dihydrocoumaroyl-CoA was not tested as substrate. Furthermore, it was noted that the sequence of the enzyme was unusually divergent from other CHS sequences (Christensen, Gregersen, Schröder, & Collinge, 1998). Hence, we did a multiple alignment (Figure 12) of the chalcone synthases used in this study and the chalcone synthase from *M. sativa*, for which the three dimensional structure was determined (Ferrer, Jez, Bowman, Dixon, & Noel, 1999). The alignment showed that HvChs2 contains two amino acid substitutions, T197A and F265I, at otherwise highly conversed residues. According to the predicted structure, the T197A substitution might affect the interaction with the carbonyl group of *p*-coumaroyl-CoA, whereas the F265I would affect the separation of the *p*-coumaroyl-binding pocket from the cyclisation pocket (Ferrer et al., 1999). Hence, these substitutions might provide some explanation for this unusual preference of *p*-dihydrocoumaroyl-CoA over *p*-coumaroyl-CoA. High substrate specificity may eventually be very valuable, enabling a more streamlined downstream process, especially in cases like this where product and by-product exhibit a high structural similarity. The lower final phloretin titer achieved in this strain is an issue that most likely can be solved, e.g. by higher copy numbers of *HvCHS2*. A similar strategy of using enzymes as filters for selective production of the compound of interest was recently used in *E. coli* for unnatural C50 carotenoids (Furubayashi et al., 2015).

```
MsChs2  MVS-----VSEIRKAQRAEGPATILAIGTANPANCVEQSTYPDFYFKITNSEHKTELKEKFQRMCDKSMIKRRYMYLTEE 75
HaChs   MVT-----VEEVRKAQRAEGPATVMAIGTAVPPNCVDQATYPDYYFRITNSEHKAELKEKFQRMCDKSQIKKRYMYLNEE 75
PcChs   MANHHNAEIEEIRNRQRAQGPANILAIGTATPSNCVYQADYPDYYFRITNSEHMTDLKLKFKRMCEKSMIRKRYMHITEE 80
PhChs   MVT-----VEEYRKAQRAEGPATVMAIGTATPTNCVDQSTYPDYYFRITNSEHKTDLKEKFKRMCEKSMIKKRYMHLTEE 75
HvChs1  MAA--TMTVEEVRNAQRAEGPATVLAIGTATPANCVYQADYPDYYFKITKSDHMADLKEKFKRMCDKSQIRKRYMHLTEE 78
HvChs2  MAA---VRLKEVRMAQRAEGLATVLAIGTAVPANCVYQATYPDYYFRVTKSEHLADLKEKFQRMCDKSMIRKRHMHLTEE 77
SbChs   MVT-----VEEFHRATRAEGPATVLAIGTANPPNCVEQSTYADYYFRICKSEHLTDLKKKFDRMCEKSCIKKRYMHLTEE 75
MdChs1  MVT-----VEEVRKAQRAEGPATVMAIGTATPSNCVDQATYPDYYFRITNSEHKVELKEKFQRMCDKSMIKKRYMYLTEE 75
MdChs2  MVT-----VEEVRKAQRAEGPATVLAIGTATPPNCVDQATYPDYYFRITNSEHKTELKEKFQRMCDKSMIKTRYMYLTEE 75

MsChs2  ILKENPNVCEYMAPSLDARQDMVVVEVPRLGKEAAVKAIKEWGQPKSKITHLIVCTTSGVDMPGADYQLTKLLGLRPYVK 155
HaChs   VLKENPNMCAYMAPSLDARQDIVVVEVPKLGKEAAVKAIKEWGQPKSKITHLVFCTTSGVDMPGADYQLTKLLGLRPSVK 155
PcChs   YLKENPNVCAYEAPSLDARQDLVVVEVPRLGKEAASKAIKEWGQPKSKITHLIFCTTSGVDMPGADYQLTKLLGLRPSVK 160
PhChs   ILKENPSMCEYMAPSLDARQDIVVVEVPKLGKEAAQKAIKEWGQPKSKITHLFFCTTSGVDMPGCDYQLTKLLGLRPSVK 155
HvChs1  ILEENPNMCAYMAPSLDARQDIVVVEVPKLGKAAAQKAIKEWGQPRSKITHLVFCTTSGVDMPGADYQLTKMLGLRPSVK 158
HvChs2  ILIKNPKICAHMETSLDARHAIALVEVPKLGQGAAEKAIKEWGQPLSKITHLVFCTTSGVDMPGADYQLTKLLGLSPTVK 157
SbChs   FLKENDNFTAYEAPSLDARQDIVVVEIPKLGKEAAQKAIKEWGQPKSKITHVIFCTTSGVDMPGADYQITKLLGLRPSVK 155
MdChs1  ILKENPSVCEYMAPSIDARQDMVVVEVPKLGKEAATKAIKEWGQPKSKITHLVFCTTSGVDMPGADYQLTKLLGLRPSVK 155
MdChs2  ILKENPTVCEYMAPSLDARQDMVVVEVPRLGKEAATKAIKEWGQPKSKITHLVFCTTSGVDMPGADYQLTKLLGLRPSVK 155

                                                  *
MsChs2  RYMMYQQGCFAGGTVLRLAKDLAENNKGARVLVVCSEVTAVTFRGPSDTHLDSLVGQALFGDGAAALIVGSDPVPEIEKP 235
HaChs   RLMMYQQGCFAGGTVLRLAKDLAENNKGARVLVVCSEITAVTFRGPTDTHLDSLVGQALFGDGAAAIIIGSDPIPEVEKP 235
PcChs   RFMMYQQGCFAGGTVLRLAKDLAENNAGARVLVVCSEITAVTFRGPSDSHLDSLVGQALFGDGAAAVILGSDPDLSVERP 240
PhChs   RLMMYQQGCFAGGTVLRLAKDLAENNKGARVLVVCSEITAVTFRGPNDTHLDSLVGQALFGDGAGAIIIGSDPIPGVERP 235
HvChs1  RLMMYQQGCFAGGTVLRLAKDLAENNRGARVLVVCSEITAVTFRGPHESHLDSLVGQALFGDGAAAVIIGADPDLSVERP 238
HvChs2  RLMMYQQGCFGGATVLRLAKDIAENNRGARVLVVCSEITAMAFRGPCKSHLDSLVGHALFGDGAAAAIIGADPDQLDEQP 237
SbChs   RFMMYQQGCFAGGTVLRMAKDLAENNAGARVLVVCSEITAITFRGPSDTHLDSLVGQALFGDGAAAVIVGSDPIVGVERP 235
MdChs1  RLMMYQQGCFAGGTVLRLAKDLAENNKGARVLVVCSEITAVTFRGPSDTHLDSLVGQALFGDGAAAVIIGADPVPEVEKP 235
MdChs2  RLMMYQQGCFAGGTVLRLAKDLAENNKGARVLVVCSEITAVTFRGPSDTHLDSLVGQALFGDGAAAVIIGSDPVPEVEKP 235

                                      *
MsChs2  IFEMVWTAQTIAPDSEGAIDGHLREAGLTFHLLKDVPGIVSKNITKALVEAFEPLGISDYNSIFWIAHPGGPAILDQVEQ 315
HaChs   LFELVSAAQTILPDSEGAIDGHLREVGLTFHLLKDVPGLISKNVEKSLTEAFKPLGISDWNSLFWIAHPGGPAILDQVEA 315
PcChs   LFQLISAAQTILPDSDGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLKEAFGPIGISDWNSLFWIAHPGGPAILDQVEL 320
PhChs   LFELVSAAQTLLPDSHGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLEEAFKPLGISDWNSLFWIAHPGGPAILDQVEI 315
HvChs1  LFQLVSASQTILPDSEGAIDGHLREVGLTFHLLKDVPGLISKNIERALEEAFKPLGIDHWNSVFWIAHQGGPAILDMVEA 318
HvChs2  VFQLVSASQTILPESEGAIDGHLTEAGLTIHLLKDVPGLISENIEQALEDAFEPLGIHNWNSIFWIAHPGGPAILDRVED 317
SbChs   LFQLVSAAQTILPDSEGAIDGHVREVGLTFHLLKDVPGLISKDIEKSLKEAFAPLGISDWNSLFWIVHPGGPAILDQVGE 315
MdChs1  LFELVSAAQTILPDSDGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLNEAFKPIGISDWNSLFWIAHPGGPAILDQVEA 315
MdChs2  LFELVSAAQTILPDSDGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLNEAFKPIGISDWNSLFWIAHPGGPAILDQVES 315

MsChs2  KLALKPEKMNATREVLSEYGNMSSACVLFILDEMRKKSTQNGLKTTGEGLEWGVLFGFGPGLTIETVVLRSV------AI 389
HaChs   KLSLKPEKLRATRHVLSEYGNMSSACVLFILDEMRRKSKEDGLKTTGEGIEWGVLFGFGPGLTVETVVLHSV------AI 389
PcChs   KLGLKEEKMRATRQVLSDYGNMSSACVLFILDEMRKKSIEEGKATTGEGLDWGVLFGFGPGLTVETVVLHSVPA----TF 396
PhChs   KLGLKPEKLKATRNVLSDYGNMSSACVLFILDEMRKASAKEGLGTTGEGLEWGVLFGFGPGLTVETVVLHSV------A- 388
HvChs1  KVNLNKERMRATRHVLSEYGNMSSACVLFIMDEMRKRSAEDGHATTGEGMDWGVLFGFGPGLTVETVVLHSVPISAG-AT 397
HvChs2  RVGLDKKRMRASREVLSEYGNMSSASVLFVLDVMRKSSAKDGLATTGEGKDWGVLFGFGPGLTVETLVLHSVPVPVPTAA 397
SbChs   KLGLKPEIMVPTRHVLSEYGNMSSACVLFVMDEMRKASAKDGCTSTGEGKDWGVLFGFGPGLTVETVVLHSVPL------ 389
MdChs1  KLALKPEKLEATRQVLSDYGNMSSACVLFILDEVRRKSAEKGLKTTGEGLEWGVLFGFGPGLTVETVVLHSV------GL 389
MdChs2  KLALKPEKLEATRQVLSDYGNMSSACVLFILDEVRRKSAEKGLKTTGEGLEWGVLFGFGPGLTVETVVLHSV------G- 388

MsChs2  --- 389
HaChs   NX- 391
PcChs   THX 399
PhChs   -TX 390
HvChs1  AXP 400
HvChs2  SAX 400
SbChs   -NX 391
MdChs1  TAX 392
MdChs2  -AX 390
```

**Figure 12.** Multiple protein sequence alignment of CHS enzymes used in this study and including MsChs2 (P51080), for which the crystal structure is known. Amino acids lining the *p*-coumaroyl binding pocket are highlighted in green, those of the cyclization pocket in cyan, and the catalytic triad in red. Of these generally highly conserved residues, T197 and F265 (marked with an asterisk, and numbered according to MsChs2) are different in HvChs2. They are interacting with the *p*-coumaroyl-CoA derived carbonyl of naringenin (T197) or separating the coumaroyl-binding site from the cyclisation pocket (F265), respectively.

### 5.1.5 Production of the monoglycosylated dihydrochalcones phlorizin and nothofagin using known UGTs

In a next step, decorating enzymes were co-expressed in a strain harboring the phloretin pathway, to evaluate the feasibility and versatility of such a strain as the basic production platform for various dihydrochalcones. Strain DBR2 was used for these experiments, not only because it produced more phloretin than strain CHS5, but also because the by-product naringenin could be used as a positive control for functional expression of decorating enzymes with activities on flavanones. First, a set of UGTs with reported activity on dihydrochalcones for production of phlorizin (phloretin 2'-*O*-glucoside) (Gosch, Halbwirth, Schneider, et al., 2010; Jugdé et al., 2008; Werner & Morgan, 2009) or nothofagin (phloretin 3'-*C*-glucoside) (Brazier-Hicks et al., 2009) were tested. The corresponding genes were expressed on a second HRT plasmid in strain DBR2, making strains PHZ1 to PHZ4 and strain NOT1. Strain UGTn with an empty second HRT plasmid was used as a negative control. The strains were cultured and production of dihydrochalcones was measured. Of the four UGTs reported to glycosylate phloretin to phlorizin, only MdUgt88a1 and PcUgt88f2 were efficiently catalyzing this reaction in our yeast strain (Figure 13A). On the other hand, OsCgt was highly efficient for nothofagin production, converting phloretin almost completely to this C-glucoside (Figure 13B).

### 5.1.6 Production of NDC using two substrate-promiscuous UGTs

Trilobatin, which is the 4'-*O*-glucoside of phloretin, is a dihydrochalcone found naturally in *Malus trilobata* and some other *Malus* species (Gosch et al., 2010b; Martens, personal communication). It is an intermediate in a proposed biosynthetic route to the sweetener NDC (Figure 4B). The equivalent position on the structurally related flavonoids is the 7-hydroxy group (Figure 4A), and AtUgt73b2 has previously been described as an enzyme with broad substrate flexibility for different flavonoid backbones (Willits et al., 2004). Two other enzymes, AtUgt84b1 and AtUgt76d1, were shown to be specific for the 7-position of quercetin (E. K. Lim, Ashford, Hou, Jackson, & Bowles, 2004). The three genes, encoding these enzymes, were expressed on a second HRT plasmid in strain DBR2, to make strains TRI1 to TRI3, and the production of dihydrochalcones was compared to the negative control strain UGTn (Figure 13C). Only AtUgt73b2 was able to glycosylate phloretin to trilobatin. Besides producing 33 ±

3 mg/l of trilobatin, it also exhibited minor 2'-*O*-glycosylation activity on phloretin to produce 1.6 ± 0.21 mg/l of phlorizin.

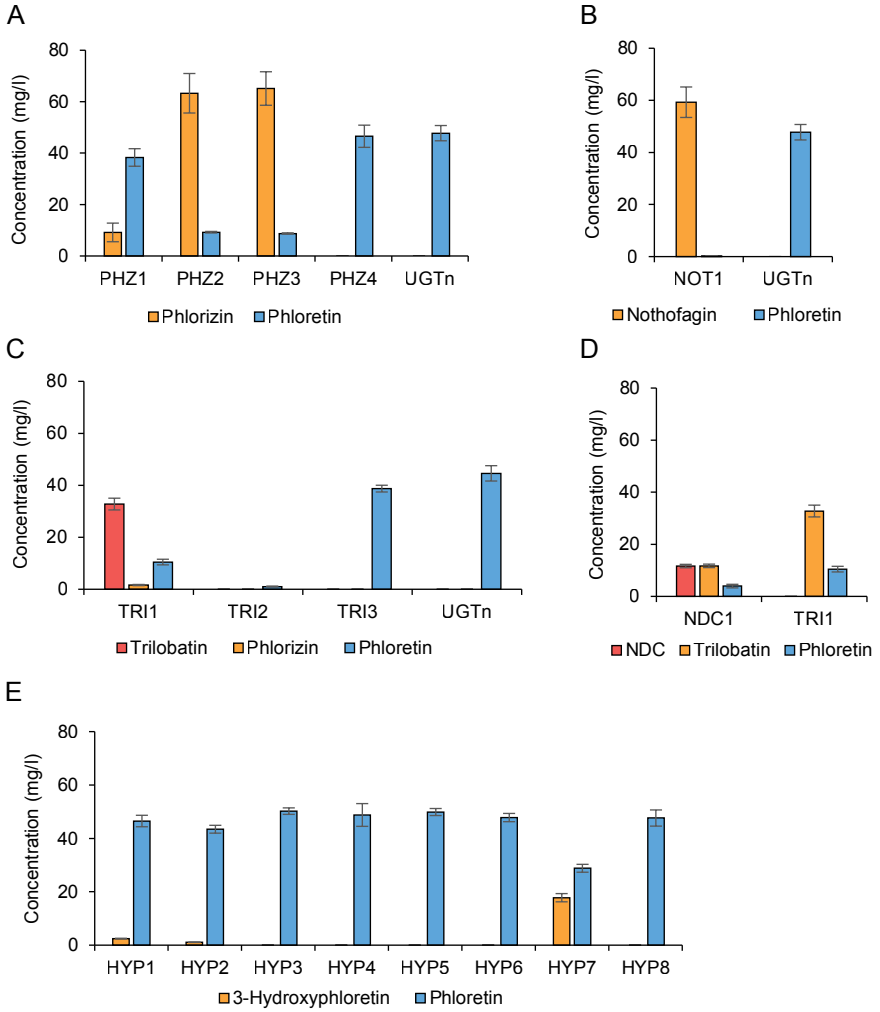


**Figure 13.** Engineering of strain DBR2 for production of various phloretin derivatives: (A) Production of phlorizin and phloretin by strains PHZ1 to PHZ4 and UGTn, harboring a second HRT plasmid, expressing different UGTs or no UGT (PHZ1: *MdUGT88F1*; PHZ2: *MdUGT88A1*; PHZ3: *PcUGT88F2*; PHZ4: *DcGT4*; UGTn: no UGT). (B) Production of nothofagin and phloretin by strains NOT1 and UGTn, harboring a second HRT plasmid, expressing either *OsCGT* (NOT1) or no enzyme (UGTn). (C) Production of trilobatin, phlorizin, and phloretin by strains TRI1 to TRI3 and UGTn, harboring a second HRT plasmid, expressing different UGTs or no UGT (TRI1: *AtUGT73B2*; TRI2: *AtUGT76D1*; TRI3: *AtUGT84B1*; UGTn: no UGT). (D) Production of naringin dihydrochalcone (NDC), trilobatin, and phloretin by strains NDC1 and TRI1, harboring a second HRT plasmid, expressing either *AtUGT73B2*, *Cm1,2RHAT*, and *AtRHM2* (NDC1) or only *AtUGT73B2* (TRI1). (E) Production of 3-hydroxyphloretin and phloretin by strains HYP1 to HYP8, harboring a second HRT plasmid, expressing different CYPs or no CYP together with *AtATR1* (HYP1: *OsF3'H*; HYP2: *PhF3'H*; HYP3: *PfF3'H*; HYP4: *AcF3'H*; HYP5: *MdF3'H1*; HYP6: *MdF3'H2*; HYP7: *CsCH3H*; HYP8: no enzyme). Represented are average and standard deviation of three independent cultures.

The next step in the proposed route to NDC is the rhamnosylation of trilobatin with an α-1,2 glycosidic bond. Cm1,2Rhat was reported to catalyze the corresponding reaction on flavanones to form naringin from naringenin 7-*O*-glucoside (Frydman et al., 2004). While UDP-rhamnose, the sugar donor required by Cm1,2Rhat, is not naturally produced by *S. cerevisiae*, its biosynthesis has been achieved by expression of *AtRHM2* from *A. thaliana* (Oka, Nemoto, & Jigami, 2007). Therefore, we expressed *AtUGT73B2*, *Cm1,2RHAT*, and *AtRHM2* on a second HRT plasmid in strain DBR2 to make strain NDC1. By comparing the production of dihydrochalcones in this strain to that of strain TRI3, which expressed only *AtUGT73B2*, we could demonstrate the ability of Cm1,2Rhat to accept trilobatin as substrate, allowing the production of 11.6 ± 0.7 mg/l of NDC (Figure 13D). A similar approach, using promiscuous enzymes, was reported in *E. coli*, where substrate flexibility of flavanone 3β-hydroxylase (F3H), flavonol synthase (FLS), and flavonoid 3'-hydroxylase (F3'H) was exploited for the assembly of a novel synthetic pathway for the 5-deoxyflavonol fisetin (Stahlhut et al., 2015). Here we adopted this approach, showing that it can be more widely applied across biosynthetic pathways. Interestingly, the enzymes which were eventually selected for constructing the various pathways, all originate from plants that have not been shown to produce dihydrochalcones.

### 5.1.7 Production of 3-hydroxyphloretin using a substrate promiscuous CYP

Many dihydrochalcones, such as 3-hydroxyphlorizin, aspalathin, and neohesperidin dihydrochalcone, contain a 3-hydroxy group (Figure 4B). The specific enzymes responsible for the biosynthesis of these compounds have not been identified, although many F3'Hs and one CH3H enzyme have been identified from various plants. These enzymes are regiospecific cytochrome P450s (CYP), which can hydroxylate the equivalent position in the structurally related flavonoids and chalcones. As they are CYPs, they require the action of a cytochrome P450 reductase (CPR) as cofactor. A collection of six F3'H from various plants, plus CsCh3h, were tested for their ability to form 3-hydroxyphloretin from phloretin in yeast. Two of the F3'H were amplified from *M.* x *domestica* var. Golden Delicious cDNA, with the rationale that they might be promiscuous towards dihydrochalcones, since apple was reported to contain 3-hydroxyphlorizin (Tsao, Yang, Young, & Zhu, 2003). Eight HRT constructs containing *AtATR1* together with one of the seven CYPs, or no CYP as negative control, were assembled in strain DBR2 to make strains HYP1 to HYP8 and production of

dihydrochalcones was measured (Figure 13E). While OsF3'h and PhF3'h showed minor activity on phloretin, CsCh3h transformed more than a third of the phloretin to 3-hydroxyphloretin. The apparent inactivity of both apple F3'H proteins is in agreement with previous experience with heterologous expression of F3'Hs from different *Malus* species, known to accumulate 3-hydroxylated dihydrochalcones, e.g. *Malus toringo syn sieboldii* (Martens, unpublished). During preparation of this manuscript another study was published, reporting the ability of CsCh3h to accept phloretin as substrate, both in yeast and in plants. Overexpression of CsCh3h in apple resulted in increased production of 3-hydroxyphlorizin, which correlated with reduced susceptibility to fire blight and scab (Hutabarat et al., 2016).

To summarize, the strain DBR2 was successfully used as a dihydrochalcone platform strain and the production of various derivatives of phloretin was achieved by using decorating enzymes with known activities on dihydrochalcone or by exploiting substrate promiscuity of enzymes naturally involved in flavonoid or chalcone biosynthesis. Table 10 gives an overview of the dihydrochalcones, for which *de novo* biosynthesis in *S. cerevisiae* was demonstrated in this proof of concept study.

**Table 10.** Titers achieved for *de novo* production of different dihydrochalcones and numbers of heterologous enzymes overexpressed for the pathways.

| Compound | Titer (mg/l) | Enzymes expressed |
|---|---|---|
| Phloretin | 42.7 ± 0.9 | 6 |
| Pinocembrin dihydrochalcone | 2.6 ± 0.3 | 4 |
| Phlorizin | 65 ± 7 | 7 |
| Nothofagin | 59 ± 6 | 7 |
| Trilobatin | 32.8 ± 2.3 | 7 |
| Naringin dihydrochalcone | 11.6 ± 0.7 | 9 |
| 3-Hydroxyphloretin | 28.8 ± 1.5 | 8 |

## 5.2 *De novo* biosynthesis of anthocyanins in *S. cerevisiae*

### 5.2.1 Pathway to naringenin and hydroxylation of the B and C rings.

The *de novo* production of naringenin in *S. cerevisiae* has been described recently, reaching titers of over 200 mg/l (Koopman et al., 2012; Lehka et al., 2017). In this work, a previously identified pathway to naringenin was used, comprising *A. thaliana PAL2*, *A. majus C4H*, *S. cerevisiae CPR1*, *A. thaliana 4CL2*, *H. androsaemum CHS*, and *M. sativa CHI* (Eichenberger et al., 2017; Lehka et al., 2017). These genes were integrated into the genome of strain BG at site XI-3 (Mikkelsen et al., 2012) using the HRT technology, resulting in strain NAR1. NAR1 was grown in DWP and production of flavonoids was quantified by UPLC-MS. Besides producing 61 ± 3 mg/l of naringenin, this strain also accumulated 82 ± 5 mg/l of phloretic acid, a by-product arising from double bond reduction of the intermediate *p*-coumaroyl-CoA by the native ScTsc13 enzyme (Lehka et al., 2017). We deemed naringenin production to be high enough for using this strain as basis for exploring the anthocyanin pathway.

One of the differentiating features of anthocyanins and flavonoids in general is the number of hydroxylations of the B-ring structure. In the case of anthocyanins, this has a major impact on color and stability of the molecule (Yonekura-Sakakibara et al., 2009). While the first hydroxyl group is derived from the polyketide starter unit *p*-coumaric acid, the second and third hydroxylation is normally performed on either the flavanone or the dihydroflavonol backbone by a group of regiospecific CYP enzymes called F3'H or F3'5'H. They belong to the same CYP75 family, with F3'H grouped into subfamily 75B and most F3'5'H grouped into subfamily 75A (Seitz, Ameres, Schlangen, Forkmann, & Halbwirth, 2015).

Three F3'H from *V. vinifera* (Bogs, Ebadi, McDavid, & Robinson, 2006), *P.* x *hybrida* (Brugliera, Barrirewell, Holton, & Mason, 1999), and *O. sativa subsp. japonica* (Shih et al., 2008) and five F3'5'H from *C. roseus* (Kaltenbach, Schröder, Schmelzer, Lutz, & Schröder, 1999), *P.* x *hybrida* (Holton et al., 1993), *O. hybrid cul* (Seitz et al., 2006), *S. lycopersicum* (Olsen et al., 2010), and *C. intybus* (Seitz et al., 2015) were selected and tested for their ability to hydroxylate naringenin *in vivo*. The genes were introduced into strain NAR1 on an HRT plasmid comprising each of the CYPs together with the CYP reductase (CPR) gene *AtCPR1* from *A. thaliana*. This reductase has previously been shown to work well with other plant CYP enzymes (Mizutani & Ohta, 1998). The resulting clones were grown in DWP and cultures were analyzed for

production of naringenin, the 3'-hydroxylated product eriodictyol, and the 3'5'-hydroxylated product 5,7,3',4',5'-pentahydroxyflavanone. The identity of 5,7,3',4',5'-pentahydroxyflavanone was inferred only from the calculated mass and the product was not absolutely quantified, since we were unable to find a supplier of a reference compound (Figure 14A). Both PhF3'h and OsF3'h showed essentially complete conversion of naringenin to eriodictyol, while PhF3'5'h, OhF3'5'h, SlF3'5'h, and CiF3'5'h almost exclusively produced 5,7,3',4',5'-pentahydroxyflavanone. Strains ERI2 and PHF4, expressing *PhF3'H* and *SlF3'5'H*, respectively, were chosen for further pathway construction.

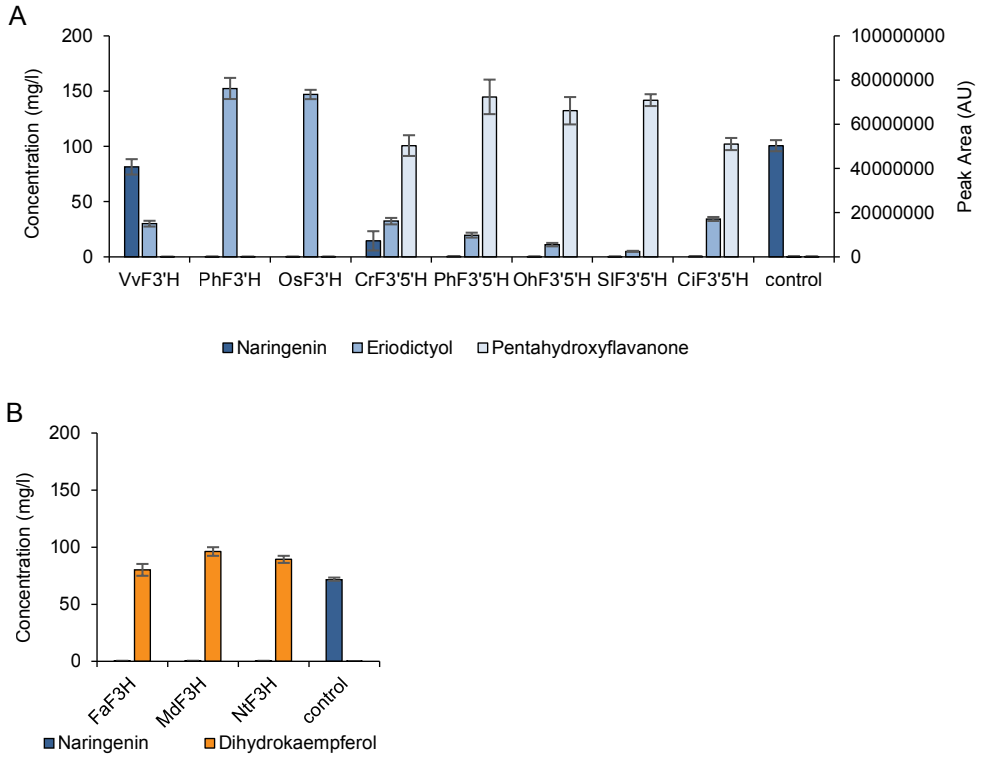


**Figure 14.** Engineering of strain NAR1 for hydroxylation of naringenin. (A) Production of naringenin, eriodictyol, and 5,7,3',4',5'-pentahydroxyflavanone by strains ERI1-ERI3 and PHF1-PHF6, expressing *AtCPR1* and different CYPs on an HRT plasmid. (B) Production of naringenin and dihydrokaempferol by strains DHK1-DHK4, expressing various F3H on an HRT plasmid. Represented are average and standard deviation of at least three independent cultures.

The next biosynthetic step in the anthocyanin pathway involves hydroxylation of the C-ring at the 3 position, converting flavanones to dihydroflavonols. This hydroxylation is catalyzed by F3H, which is a 2ODD enzyme (Martens, Preuß, & Matern, 2010). Three F3H enzymes from *F.* x *ananassa* (Almeida et al., 2007), *M.* x *domestica* (Davies, 1993), and *N. tabacum* (GenBank; direct submission) were

compared for their efficiency at hydroxylating naringenin in yeast. Hence, they were expressed on an HRT plasmid in the naringenin producing strain NAR1 and production of flavonoids was quantified. All three enzymes showed essentially complete conversion of naringenin to dihydrokaempferol (Figure 14B). MdF3h, which was previously used in *E. coli* for the production of anthocyanins (Yan, Chemler, et al., 2005; Yan et al., 2008), was chosen for further pathway construction.

### 5.2.2 DFRs are efficient in the biosynthetic pathways to flavan-3-ols

The next reaction towards anthocyanins is catalyzed by DFR, which reduces the 4-ketogroup of dihydroflavonols to form the corresponding leucoanthocyanidins. DFRs can have a significant influence on flower color by exhibiting different substrate preferences for dihydroflavonols with different hydroxylation patterns on the B-ring (Forkmann & Heller, 1999). While many DFRs catalyze the reduction of all three major dihydroflavonols, DFRs from certain species such as Petunia or *Cymbidium hybrida* do not accept dihydrokaempferol and these plants therefore do not accumulate pelargonidin-type anthocyanins. (Johnson et al., 2001; Xie, Jackson, Cooper, Ferreira, & Paiva, 2004).

Leucoanthocyanidins, the products of DFR, as well as anthocyanidins, the products of the next step in the anthocyanin pathway, are both chemically unstable (Nakajima, Tanaka, Yamazaki, & Saito, 2001; Stafford & Lester, 1984). Therefore, we decided to deviate from the linear anthocyanin pathway and instead direct it towards the more stable flavan-3-ols, in order to test the efficiency and substrate preference of DFR enzymes. Leucoanthocyanidin reductase (LAR) catalyzes the removal of the 4-hydroxygroup of leucoanthocyanidins to form corresponding flavan-3-ols (Tanner et al., 2003). We therefore tested the ability of five different DFR enzymes to drive flavan-3-ol production by co-expressing a LAR enzyme from *V. vinifera* with activity on all three basic leucoanthocyanidins (Maugé et al., 2010). Two DFR enzymes, from *A. andraeanum* (Leonard et al., 2008) and from *M.* x *domestica* (Fischer, Halbwirth, Meisel, Stich, & Forkmann, 2003), were chosen for their reported activity on dihydrokaempferol. Additionally, we selected three other DFRs, with reported activity on dihydroquercetin or dihydromyricetin, from *A. thaliana* (Leonard et al., 2008), *P. trichocarpa* (Y. Huang et al., 2012), and *I. hollandica* (Katsumoto et al., 2007).

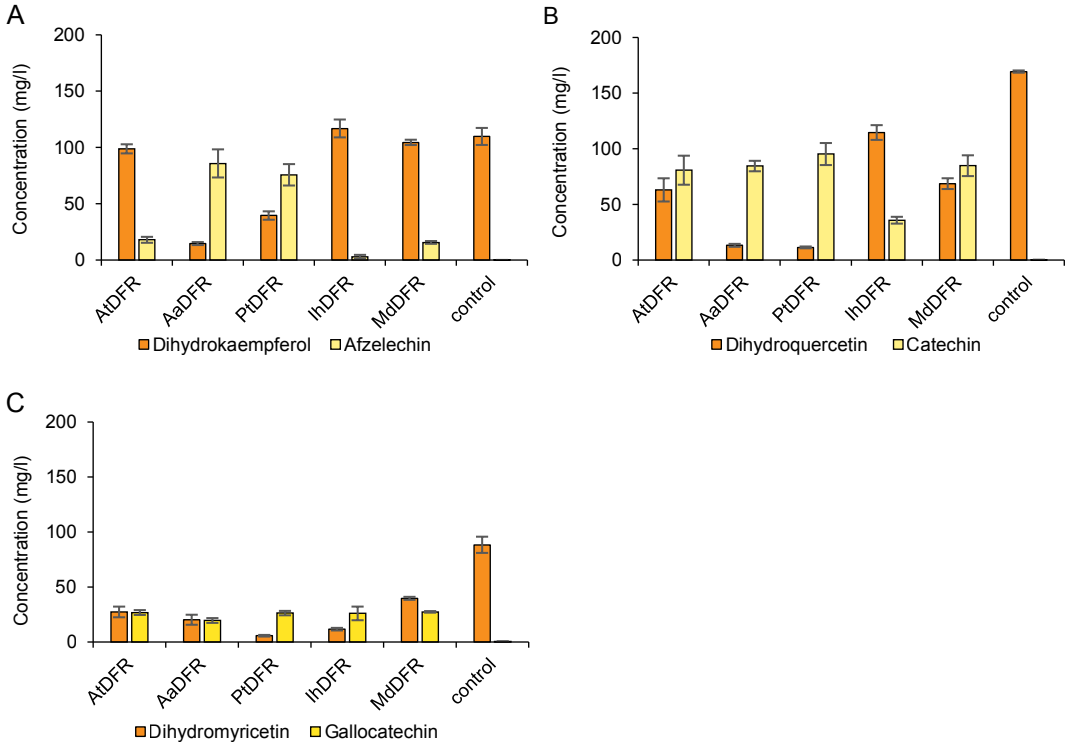


**Figure 15.** Testing DFRs in pathways to flavan-3-ols. (A) Production of dihydrokaempferol and afzelechin by strains AFZ1-AFZ6, prepared by expressing *MdF3H*, *VvLAR*, and different DFRs on an HRT plasmid in strain NAR2. (B) Production of dihydroquercetin and catechin by strains CAT1-CAT6, prepared by expressing *MdF3H*, *VvLAR*, and different DFRs on an HRT plasmid in strain ERI2. (C) Production of dihydromyricetin and gallocatechin by strains GAL1-GAL6, prepared by expressing *MdF3H*, *VvLAR*, and different DFRs on an HRT plasmid in strain PHF4. Represented are average and standard deviation of at least three independent cultures.

In order to test these DFRs we used strains NAR2, ERI2, and PHF4, producing naringenin, eriodictyol, or 5,7,3',4',5'-pentahydroxyflavanone, respectively. Strain NAR2 was obtained by transforming the NAR1 strain with an empty plasmid harboring the same LEU2 auxotrophic marker that was used to create the ERI2 and PHF4 strains. The three strains were transformed with a second HRT plasmid containing *MdF3H* and *VvLAR* in combination with one of the five DFR genes, or no DFR as negative control. All resulting strains were grown and analyzed for production of flavonoids (Figure 15). Dihydrokaempferol was accepted by four of the five enzymes with varying efficiency. Besides the previously reported AaDfr and MdDfr, also AtDfr and PtDfr accepted dihydrokaempferol, resulting in afzelechin production. AaDfr gave the highest afzelechin titer and was therefore chosen for further pathway construction towards pelargonidin-3-O-glucoside. All DFRs tested resulted in similar titers of catechin and gallocatechin, except for IhDfr, which had a lower efficiency on dihydroquercetin, as

was previously reported (Katsumoto et al., 2007). For pathway construction towards cyanidin-3-O-glucoside we chose PtDfr, giving the highest catechin titer, while for delphinidin-3-O-glucoside we selected IhDfr, as it was most specific for reducing dihydromyricetin.

### 5.2.3 Biosynthesis of anthocyanins and testing of A3GTs

Having established efficient pathways to all three flavan-3-ols, we attempted to complete the heterologous pathway to anthocyanins. ANS catalyzes the oxidation of leucoanthocyanidins, resulting in the unstable anthocyanidin intermediates, which are then stabilized by glycosylation of the 3-OH position by A3GT. *In vitro*, ANS was previously shown to oxidize leucoanthocyanidins to mainly flavonols, probably through a second oxidation on the C-4, with anthocyanidins being only a minor product (Turnbull et al., 2003). When dihydroflavonols were used as substrate, ANS exhibited flavonol synthase (FLS) activity, resulting in flavonol production (Turnbull et al., 2000). Furthermore, it was shown that anthocyanidins can result from oxidation of flavan-3-ols by ANS *in vitro*. When A3GT was co-expressed with ANS, the product profile profoundly shifted from flavan-3-ol dimers towards anthocyanins (Wellmann et al., 2006). Similarly, when the pathway from flavanones to anthocyanins was reconstituted in *E. coli*, presence of efficient A3GT activity was crucial for accumulation of anthocyanins (Yan et al., 2008).

Therefore, we first concentrated on testing a collection of nine A3GTs from various plant origins for production of the three core anthocyanins, hoping they would stabilize the unstable anthocyanidin intermediates. Sequences were derived from *O. sativa subsp. japonica* (Ko et al., 2008), *G. triflora* (Tanaka et al., 1996; Ueyama, Suzuki, Fukuchi-Mizutani, & Fukui, 2002), *P.* x *hybrida* (Yamazaki et al., 2002), *A. thaliana* (E. K. Lim et al., 2004), *F.* x *ananassa* (Almeida et al., 2007; Griesser et al., 2008), *V. vinifera* (Ford, Boss, & Høj, 1998), and *D. caryophyllus* (Ogata et al., 2004). In order to have a stable expression system for these experiments, we performed a second integration in strain NAR1 at integration site XI-2 (Mikkelsen et al., 2012), completing the pathways to the anthocyanidins pelargonidin, cyanidin, and delphinidin.

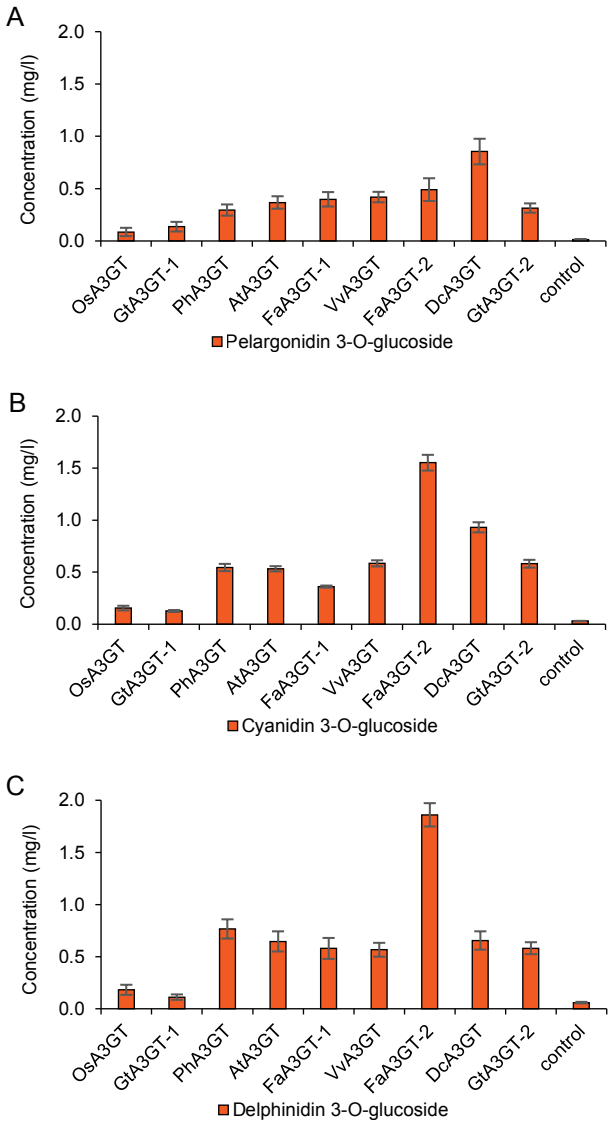


**Figure 16.** Testing A3GTs in pathways to anthocyanins. (A) Production of pelargonidin-3-O-glucoside by strains PGT1-PGT10, prepared by expressing different A3GTs on an expression plasmid in strain PEL1. (B) Production of cyanidin-3-O-glucoside by strains CGT1-CGT10, prepared by expressing different A3GTs on an expression plasmid in strain CYA1. (C) Production of delphinidin-3-O-glucoside by strains DGT1-DGT10, prepared by expressing different A3GTs on an expression plasmid in strain DEL1. Represented are average and standard deviation of at least three independent cultures.

All three pathways included the *MdF3H* and *PhANS*, both of which had previously been used for reconstitution of anthocyanin pathways in *E. coli* (Yan, Chemler, et al., 2005; Yan et al., 2008). Additionally, the pathways contained the best DFR for each hydroxylation pattern (*AaDFR* for pelargonidin, *PtDFR* for cyanidin, and *IhDFR* for delphinidin), and *AtCPR1* together with *PhF3'H* or *SlF3'5'H* for cyanidin and delphinidin, respectively. HRT technology was used to integrate these genes into NAR1, resulting in strains PEL1, CYA1, and DEL1. The A3GTs were then introduced

on single copy expression plasmids and resulting strains were analyzed for production of anthocyanins (Figure 16). Anthocyanidins were not quantified, due to their instability. The choice of A3GT had a big influence on accumulation of the anthocyanins, with DcA3gt giving the highest titer of 0.85 ± 0.12 mg/l for pelargonidin-3-O-glucoside, and FaA3gt2 being most efficient for cyanidin-3-O-glucoside and delphinidin-3-O-glucoside, yielding 1.55 ± 0.08 mg/l and 1.86 ± 0.11 mg/l, respectively. Besides production of anthocyanins, we also observed accumulation in all strains of dihydroflavonols, flavonols, and flavonol-3-O-glucosides above the linear range of our calibration curve.

### 5.2.4 Production of flavonols is a common trait of ANS enzymes in yeast

Our yeast strains, engineered for production of anthocyanins, accumulated high amounts of dihydroflavonol and flavonol by-products. In order to test, whether this is a specific effect of PhAns used in the pathways, or whether this is a more general trait of all ANS enzymes, we tested a collection of 14 ANS originating from a wide range of plants (*I. nil* (GenBank; direct submission), *G.* x *hybrida* (Wellmann et al., 2006), *M.* x *domestica* (Honda et al., 2002), *P.* x *hybrida* (Weiss, van der Luit, Kroon, Mol, & Kooter, 1993), *F.* x *ananassa* (Almeida et al., 2007), *P. communis* (Fischer et al., 2007), *I. batatas* (Liu et al., 2010), *S. tuberosum* (GenBank ; direct submission), *M. sprengeri* (Shi, Li, Kang, & Liu, 2015), *G. biloba* (Xu et al., 2008), *Z. mays* (Menssen et al., 1990), *A. thaliana* (Pelletier, Murrell, & Shirley, 1997), *O. sativa subsp. Indica* (Reddy, Reddy, Scheffler, Wienand, & Reddy, 2007), and *A. cepa* (Kim, Binzel, Yoo, Park, & Pike, 2004)). Strains NAR2, ERI2, and PHF4, producing either naringenin, eriodictyol, or 5,7,3',4',5'-pentahydroxyflavanone, respectively, were provided with a second HRT plasmid, containing *MdF3H*, the best DFR (*AaDFR* for pelargonidin-3-O-glucoside, *PtDFR* for cyanidin-3-O-glucoside, and *IhDFR* for delphinidin-3-O-glucoside) and A3GT (*DcA3GT* for pelargonidin-3-O-glucoside, *FaA3GT2* for cyanidin-3-O-glucoside and delphinidin-3-O-glucoside) for each hydroxylation pattern, and one of the 14 different ANS or an empty expression cassette as negative control. Production of anthocyanins and flavonol by-products was quantified for the resulting strains (Figure 17, Figure 18). This revealed some ANS dependent variation in the production of anthocyanins. However, even with the better enzymes we saw an increase of only about two fold compared to the PhAns used previously.

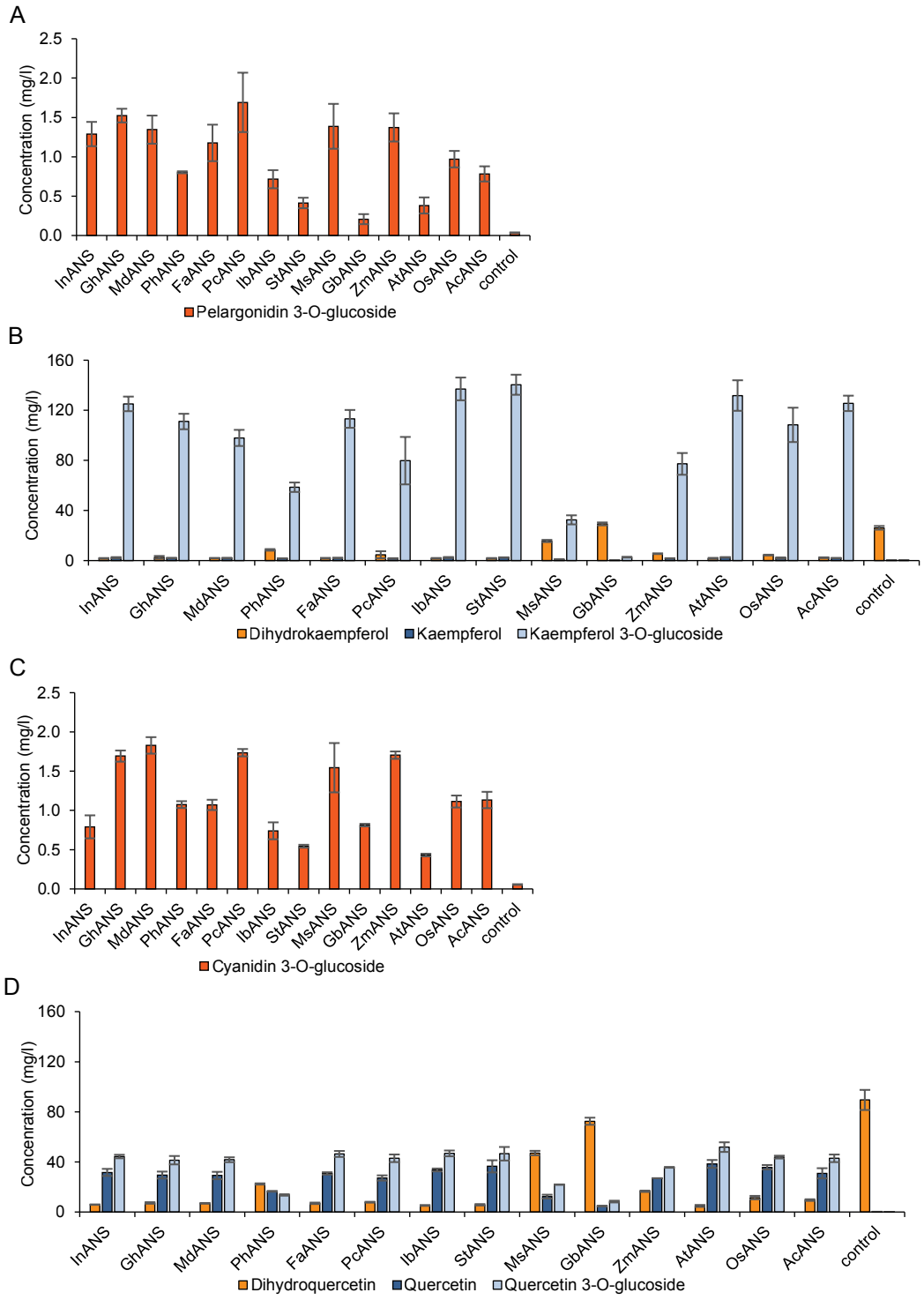


**Figure 17.** Testing ANS genes in pathways to pelargonidin-3-O-glucoside and cyanidin-3-O-glucoside. (A,B) Production of pelargonidin-3-O-glucoside, dihydrokaempferol, kaempferol, and kaempferol-3-O-glucoside by strains PANS1-PANS15, prepared by expressing *DcA3GT*, *MdF3H*, *AaDFR*, and different ANSs on an HRT plasmid in strain NAR2. (C,D) Production of cyanidin-3-O-glucoside, dihydroquercetin, quercetin, and quercetin-3-O-glucoside by strains CANS1-CANS15, prepared by expressing *FaA3GT2*, *MdF3H*, *PtDFR*, and different ANSs on an HRT plasmid in strain ERI2.

On the other hand, differences in the production of flavonol by-products were more distinct. Most ANS tested resulted in flavonol or flavonol-3-O-glucoside by-product formation roughly two orders of magnitude higher than anthocyanin production. However some ANS, mainly MsAns and GbAns, resulted in accumulation of dihydroflavonol intermediates instead of flavonol by-products.

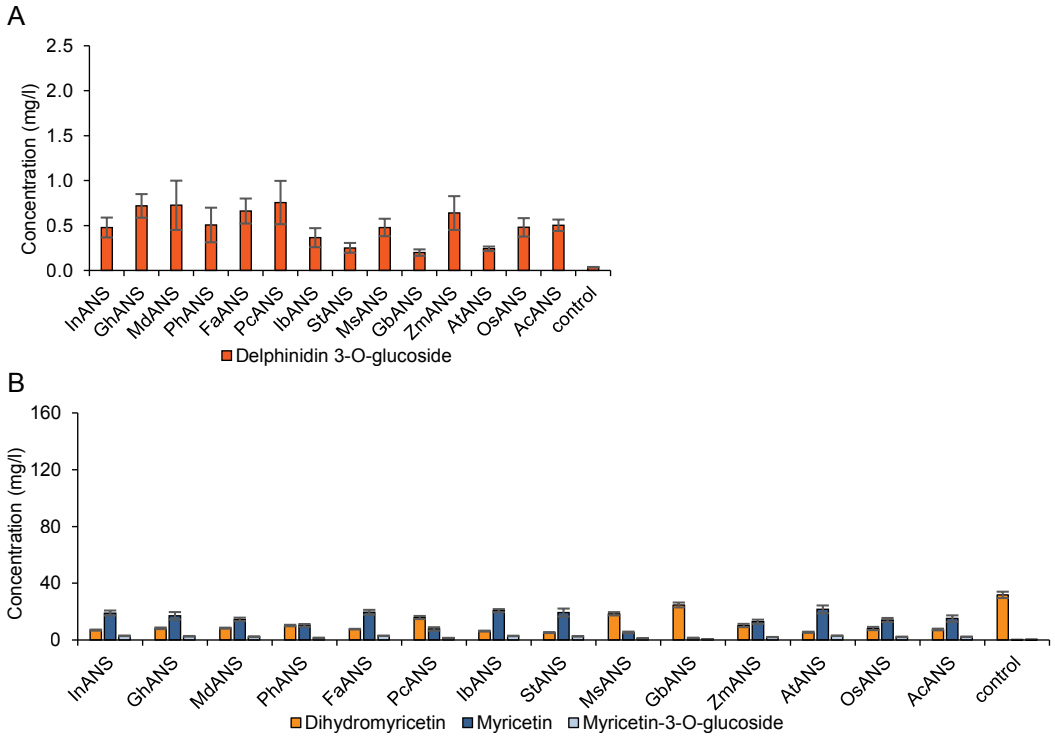


**Figure 18.** Testing ANS genes in pathways to delphinidin-3-O-glucoside. (A,B) Production of delphinidin-3-O-glucoside, dihydromyricetin, myricetin, and myricetin-3-O-glucoside by strains DANS1-DANS15, prepared by expressing *FaA3GT2*, *MdF3H*, *IhDFR*, and different ANSs on an HRT plasmid in strain PHF4. Represented are average and standard deviation of at least three independent cultures.

### 5.2.5 *In vivo* flavonol production by ANS in yeast

In order to better understand the formation of flavonol by-products in yeast, we investigated in more detail whether this was a result of FLS activity of ANS on dihydroflavonols, or due to a second oxidation of the flav-2-en-3,4-diol intermediate derived from leucoanthocyanidins. Both has been proposed for *in vitro* reactions with ANS (Turnbull et al., 2000, 2003). Additionally, ANS has been shown to catalyze also oxidation of flavan-3-ols to anthocyanidins *in vitro* (Wellmann et al., 2006). Based on this observation Yan and coworkers added a LAR to the heterologous anthocyanin pathway in *E. coli*, resulting in a boost of anthocyanin production (Yan et al., 2008).

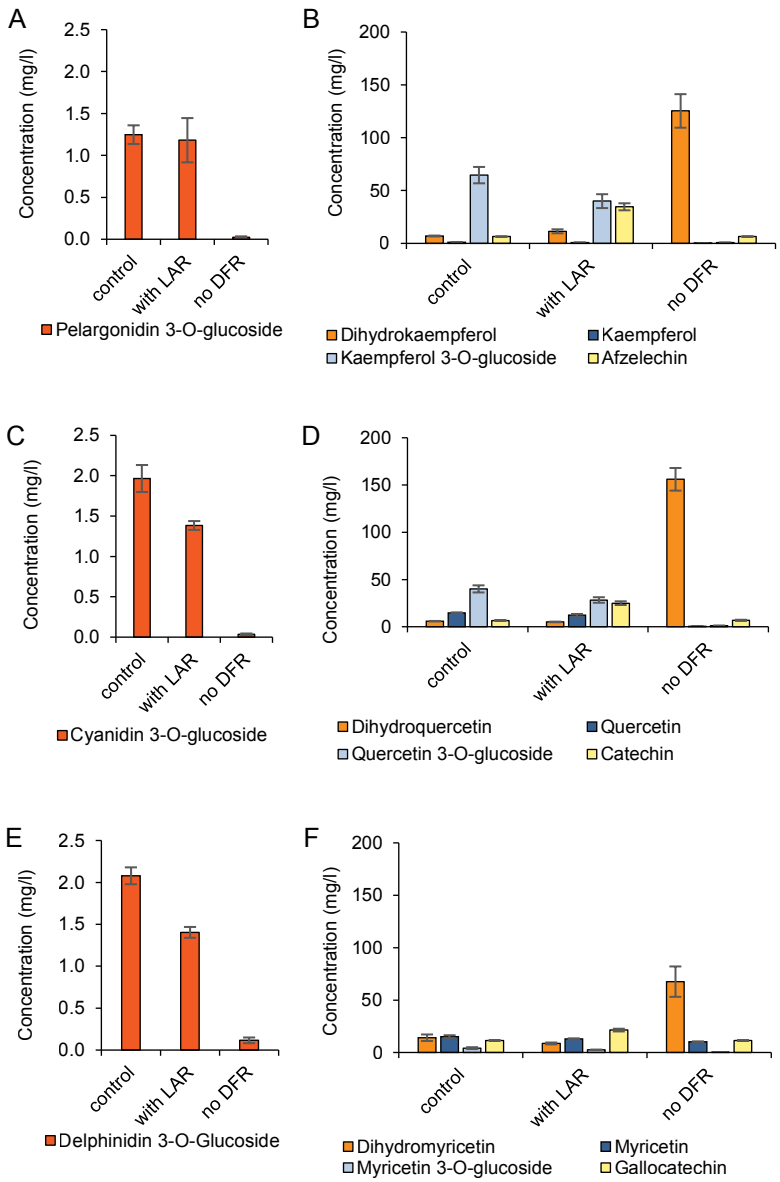


**Figure 19.** Testing different anthocyanin pathway variants. For all three anthocyanins, the linear pathway (control) was compared to the same pathway additionally including *VvLAR* (with LAR), and to the linear pathway without DFR. (A,B) Production of pelargonidin-3-O-glucoside, dihydrokaempferol, kaempferol, and kaempferol-3-O-glucoside by strains PANS6 (control), PDFR1 (with LAR), and PDFR2 (no DFR). (C,D) Production of cyanidin-3-O-glucoside, dihydroquercetin, quercetin, and quercetin-3-O-glucoside by strains CANS3 (control), CDFR1 (with LAR), and CDFR2 (no DFR). (E,F) Production of delphinidin-3-O-glucoside, dihydromyricetin, myricetin, and myricetin-3-O-glucoside by strains DANS6 (control), DDFR1 (with LAR), and DDFR2 (no DFR). Represented are average and standard deviation of at least three independent cultures.

In order to address these observations, we created both partial and extended pathways to the three anthocyanins. We constructed the best pathways, corresponding to each of the three hydroxylation patterns, consisting of *MdF3H*, the best DFR and A3GT as above, and the best ANS (*MdANS* for cyanidin-3-O-glucoside, and *PcANS* for

pelargonidin-3-O-glucoside and delphinidin-3-O-glucoside). For each of those three hydroxylation patterns we assembled three pathways of different length on a second HRT plasmid in strains NAR2, ERI2, and PHF4: First the linear full length anthocyanin pathway as control, second the same pathway but without DFR, and third the full length pathway with addition of *VvLAR*. The aim of this setup was to provide some more information about the *in vivo* substrate preference of ANS. The nine resulting strains were cultivated and production of anthocyanins and flavonol by-products was quantified (Figure 19). When leaving out DFR, accumulation of flavonol and flavonol-3-O-glucoside was drastically reduced, indicating low affinity of ANS for dihydroflavonols. Addition of VvLar to the pathways did not result in any increase in anthocyanin production, unlike what was found in *E. coli* (Yan et al., 2008). While the reported increase in *E. coli* was about 10%, our observation here would suggest that in yeast flavan-3-ols are not accepted as substrate by ANS. This was further supported by the fact that when feeding flavan-3-ols to strains expressing ANS and A3GT enzymes, no conversion to anthocyanins was observed (data not shown).

### 5.2.6 Discussion

We report here the successful reconstitution of the full pathways to pelargonidin-3-O-glucoside, cyanidin-3-O-glucoside, and delphinidin-3-O-glucoside in *S. cerevisiae*. This marks the first time anthocyanins are synthesized from glucose in a single microorganism. The titers achieved are lower than what was previously reported for pelargonidin-3-O-glucoside in *E. coli* polyculture (Jones et al., 2017). However, the yeast strain used in our study has not yet been specifically optimized for providing the relevant precursors, e.g. phenylalanine or acetyl- and malonyl-CoA, nor have we performed any extensive optimization of culturing conditions. Hence, the current titers could no doubt be substantially improved, and we believe to have demonstrated that yeast has the potential for efficient anthocyanin production. Reconstitution of the full pathway in a single microbial host will allow development and scale up of stable fermentation processes, starting from glucose or other cheap raw materials.

The efficient hydroxylation of naringenin by F3'H and F3'5'H demonstrates the superior ability of *S. cerevisiae* to functionally express plant CYPs. The titer of 152 ± 10 mg/l of eriodictyol from glucose achieved here is within the same order of magnitude as the highest titers reported in *E. coli* by precursor feeding of phenylalanine (107 mg/l) (Zhu, Wu, Du, Zhou, & Chen, 2014) or caffeic acid (150 mg/l) (Fowler,

Gikandi, & Koffas, 2009) and exceeds previously reported titers (6.5 mg/l) for *de novo* biosynthesis in *S. cerevisiae* (Yan, Kohli, & Koffas, 2005). Heterologous production of 5,7,3',4',5'-pentahydroxyflavanone has to the best of our knowledge not been reported in microorganisms.

It has been suggested that DFR might represent a rate limiting step in the anthocyanin pathway (Y. Huang et al., 2012; Rosati, Cadic, Duron, Renou, & Simoneau, 1997; Yan et al., 2008). Here, we tested the activity of DFR indirectly, by branching off the pathway towards flavan-3-ols by including a LAR enzyme. Some DFRs resulted in almost complete conversion of dihydroflavonols to flavan-3-ols via the instable leucoanthocyanidin intermediate, demonstrating a high catalytic activity of both enzymes. Compared to our results, previously reported titers of F30s are about one order of magnitude higher, although this was achieved by feeding with eriodictyol (S. Zhao et al., 2015). For afzelechin we show more than triple the recently reported titers for *de novo* biosynthesis (26.1 mg/l), obtained in a co-culture system of three engineered *E. coli* strains (Jones et al., 2017). For both catechin and gallocatechin we believe the heterologous biosynthesis from glucose has not been reported before. Hence, these results provide new information, which could be used in the study of proanthocyanidins, for which flavan-3-ols are the precursors. Proanthocyanidins, are the most commonly consumed tannins in the American diet and have been linked to a large number of health benefits (Aron & Kennedy, 2008). However, the polymerization mechanisms of these compounds are still not entirely clear, and production of the various precursors in yeast may facilitate the further study of these mechanisms.

A remaining obstacle in the production of anthocyanins in yeast is the observed accumulation of flavonols and their glycosides. Our results indicate that these by-products may be the result of ANS performing a second oxidation of leucoanthocyanidin, the preferred substrate, via a flav-2-en-3,4-diol intermediate as has previously been suggested by *in vitro* experiments (Turnbull et al., 2003). A direct FLS-like activity of ANS on dihydroflavonols was much less pronounced. For commercial scale production of anthocyanins, such by-product formation is obviously an issue, as it results in carbon loss. One way to circumvent the issue was recently demonstrated by Jones and co-workers who used a polyculture of *E. coli*, in which one strain produces a flavan-3-ol substrate, in turn used by a second strain to produce pelargonidin-3-O-glucoside (Jones et al., 2017). By this strategy the ANS enzyme does not encounter the leucoanthocyanidin substrate, and in *E. coli* ANS is able to convert flavan-3-ols to

anthocyanidins. In our experiments, adding LAR to the full length pathway did not give any increase in anthocyanin, and we failed to produce anthocyanin when a strain, only expressing ANS and A3GT, was fed with catechin (data not shown). A complete lack of ability by yeast to take up catechin cannot be ruled out, but we find this unlikely when comparing to similar flavonoids that are readily taken up. Hence, we rather interpret the results as the inability of ANS to use this substrate in yeast.

An alternative strategy would be engineering of ANS, by random or rational mutagenesis, to prevent the second oxidation cycle, e.g. by direct interference with the catalytic site or by forcing early release of the substrate. For any such approach a screening assay would be able to take advantage of the colour development correlated to the production of anthocyanin.

In light of the observed ANS activity it remains an intriguing question how anthocyanin biosynthesis occurs in plants. As already mentioned, the predominant products of ANS, in *in vitro* experiments, and in both *E. coli* and S. cerevisiae, are the flavonols and flavonol-3-O-glucosides (Turnbull et al., 2003; Yan et al., 2008). But, although ANS has been shown to produce small amounts of flavonols also in plants, its main function *in vivo* is production of anthocyanins (Owens et al., 2008; Stracke et al., 2009). One hypothesis is that plants achieve this by organizing the anthocyanin biosynthetic enzymes in a metabolon, something which has already been suggested for the early flavonoid pathway (Burbulis & Winkel-Shirley, 1999; Crosby, Pietraszewska-Bogiel, Gadella, & Winkel, 2011; Laursen, Møller, & Bassard, 2015). While ANS and A3GT have not been shown to interact directly with each other, the formation of a metabolon might allow channeling of the flav-2-en-3,4-diol intermediate to A3GT, thereby forming the more stable glycoside (Nakajima et al., 2001; Turnbull et al., 2003).

Another hypothesis is linked to the transport of anthocyanins in plants, where these compounds accumulate in the vacuole. Transporters belonging to the ABC or MATE families were suggested to transport anthocyanins into the vacuole (Francisco et al., 2013; Marinova et al., 2007; J. Zhao, 2015; J. Zhao & Dixon, 2010). Additionally, glutathione-S-transferases (GSTs) are thought to take part in anthocyanin transport as cytosolic carrier proteins (Mueller et al., 2000; Sun et al., 2012). Because plants mutated in transporters or GSTs have a decreased anthocyanin content, vacuolar transport is thought to be essential for their production (Alfenito et al., 1998; Goodman et al., 2004; Kitamura et al., 2016, 2004; Sun et al., 2012; Yamazaki et al., 2008). Reconstitution of plant transporter systems in a simpler, unicellular host would facilitate

the study of their individual parts, and help shed more light on the interplay between these components. In this respect, *S. cerevisiae* would be a good choice of host, and the activity of several plant anthocyanin transporters have already been studied in yeast microsomal fractions (Francisco et al., 2013; Marinova et al., 2007; J. Zhao & Dixon, 2009). Combining this work with the heterologous biosynthesis of anthocyanins would provide a powerful setup for studying anthocyanin transport. Ideally, this should take an interdisciplinary approach, including structural-, plant-, systems- and synthetic biology in order to elucidate the secrets of anthocyanin biosynthesis of plants. Eventually, this should also allow the advance, from this proof of concept study towards single host production of specifically designed anthocyanins at commercially interesting levels.

## 5.3 GSTs allow anthocyanin production without flavonol accumulation

### 5.3.1 Testing GSTs in pathways to the three core anthocyanins

Glutathione-S-transferases (GSTs) are thought to be involved in vacuolar transport of anthocyanins in plants (J. Zhao, 2015). Mutations in GSTs *bronze2* of *Z. mays*, *an9* of *Petunia hybrida*, *fl3* in *D. caryophyllus* L., and *tt19* of *A. thaliana* all result in plants with heavily reduced anthocyanin content (Alfenito et al., 1998; Goodman et al., 2004; Kitamura et al., 2004; Larsen, Alfenito, Briggs, & Walbot, 2003). We hypothesized that this might be due to an involvement of GSTs in efficient anthocyanidin formation by ANS. *S. cerevisiae* strains PEL2, CYA2, and DEL2, producing the anthocyanidins pelargonidin, cyanidin, and delphinidin, respectively, were used as test bed orthogonal to plants. A collection of GSTs was examined for their effect on anthocyanin accumulation in these three yeast strains. This included six GSTs previously shown to be involved in anthocyanin production by complementation of the above plant mutants: ZmBz2 (Marrs, Alfenito, Lloyd, & Walbot, 1995), PhAn9 (Alfenito et al., 1998), AtTt19 (A. P. Smith et al., 2003), VvGst4 (Conn, Curtin, Bézier, Franco, & Zhang, 2008), PfGst1 (Yamazaki et al., 2008), and CsGst3 (Kitamura, Akita, Ishizaka, Narumi, & Tanaka, 2012). Two additional variants of *A. thaliana* AtTt19 were also tested, AtTt19-3 (Kitamura et al., 2004) and AtTt19-4 (X. Li et al., 2011), of which only the latter could complement the phenotype of decreased anthocyanin accumulation. VvGst1, VvGst2, VvGst3, and VvGst5 were selected as negative controls for VvGst4, because they were reported to not complement the low abundance of anthocyanins in *bronze-2*-deficient corn kernels or *tt19-1 A. thaliana* plants (Conn et al., 2008; Pérez-Díaz, Madrid-Espinoza, Salinas-Cornejo, González-Villanueva, & Ruiz-Lara, 2016). Finally TaGstl1 was selected as a GST reported to bind flavonoids, which had not been tested in the context of an anthocyanin pathway before (Dixon & Edwards, 2010).

Plasmids expressing the coding sequence of these GSTs, or an empty plasmid as control (control 1), were co-transformed with plasmids expressing the optimal A3GT for glycosylating each of the anthocyanidins (*DcA3GT* for pelargonidin and *FaA3GT2* for cyanidin and delphinidin), and an empty *URA3* expression plasmid. Two additional control strains were generated to test the effect of GSTs without A3GT: Control 2, where a plasmid expressing *PhAN9* was co-transformed with two empty plasmids containing a *URA3* and *LEU2* auxotrophic marker, and control 3, containing three

empty plasmids with auxotrophic markers for URA, HIS, and LEU. Production of anthocyanins and flavonols was analyzed after 72 hours of growth (Figure 20).

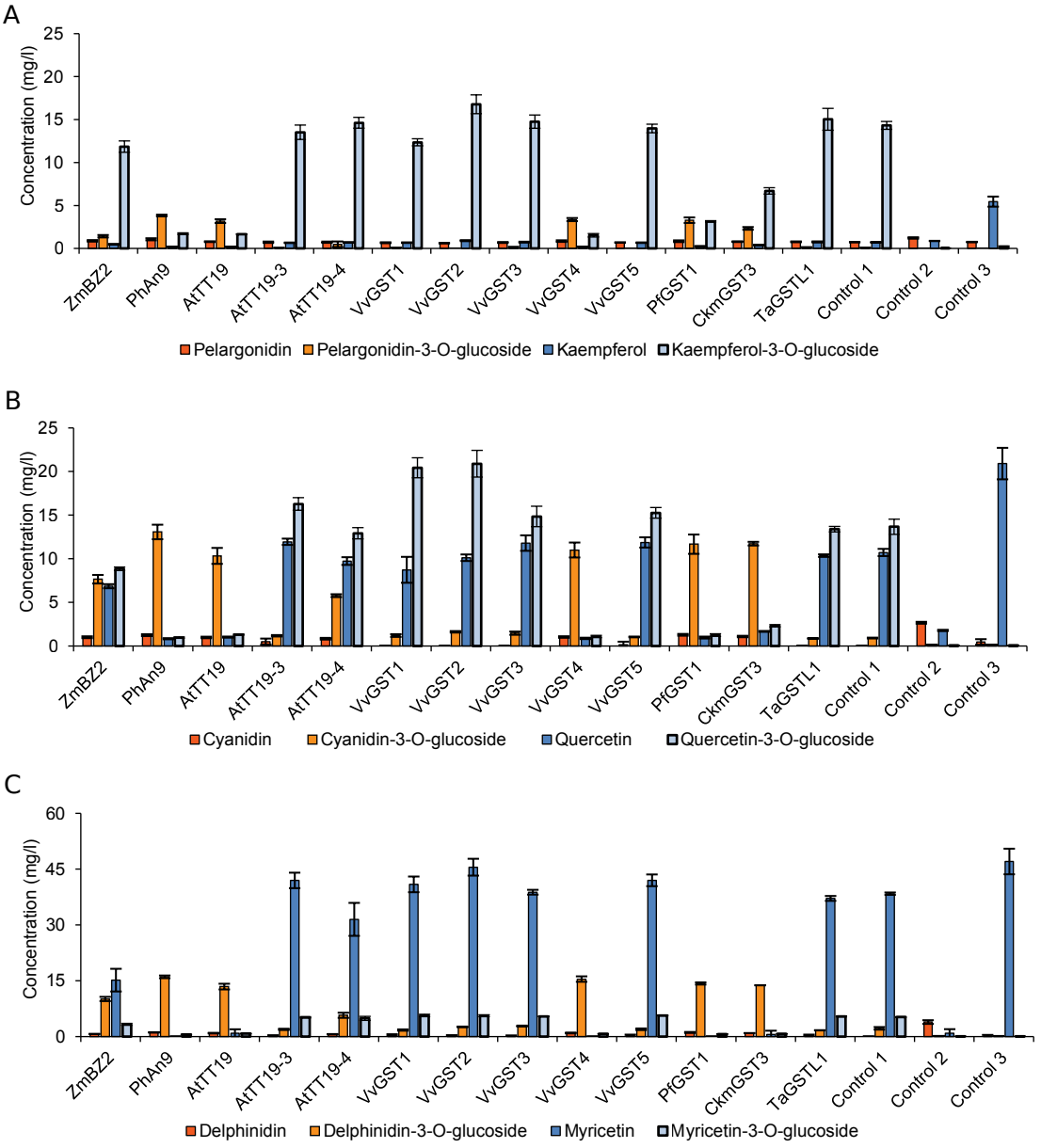


**Figure 20.** Testing GSTs in pathways to anthocyanins. (A) Production of pelargonidin, pelargonidin-3-O-glucoside, kaempferol, and kaempferol-3-O-glucoside of strains PGST1-PGST16, co-expressing different GSTs with *DcA3GT*, expressing only *DcA3GT* (Control 1), expressing only *PhAN9* (Control 2), or harboring empty plasmids (Control 3) in strain PEL2. (B) Production of cyanidin, cyanidin-3-O-glucoside, quercetin, and quercetin-3-O-glucoside of strains CGST1-CGST16, co-expressing different GSTs with *FaA3GT2*, expressing only *FaA3GT2* (Control 1), expressing only *PhAN9* (Control 2), or harboring empty plasmids (Control 3) in strain CYA2. (C) Production of delphinidin, delphinidin-3-O-glucoside, myricetin, and myricetin-3-O-glucoside of strains DGST1-DGST16, co-expressing different GSTs with *FaA3GT2*, expressing only *FaA3GT2* (Control 1), expressing only *PhAN9* (Control 2), or harboring empty plasmids (Control 3) in strain DEL2. Represented are average and standard deviation of at least three independent cultures.

All seven GSTs previously shown to complement the low anthocyanin accumulation of plant mutants, i.e. ZmBz2, PhAn9, AtTt19, AtTt19-4, VvGst4, PfGst1, and CkmGst3, resulted in an increased production of anthocyanins and a reduction in flavonol by-product formation. The efficiency varied between GSTs and depended on the anthocyanin hydroxylation pattern. The six other GSTs tested had none of these effects. Even without co-expression of an A3GT (control 2) the overexpression of PhAn9 resulted in accumulation of small amounts of the unstable anthocyanidins, with flavonol accumulation being heavily reduced.

### 5.3.2 Expression of plant transporters has no effect on anthocyanin production in yeast

A range of different transporters belonging to the ABC and MATE families have been linked to anthocyanin formation in plants (J. Zhao & Dixon, 2010). Like for GSTs, mutations in such transporters result in reduced accumulation of anthocyanins (Goodman et al., 2004; J. Zhao et al., 2011). Therefore, yeast was used in order to test the effects of co-expressing plant transporters with a functional pathway to cyanidin-3-O-glucoside. The selection of transporters included 11 MATE transporters suggested to be involved in anthocyanin transport: AtTt12 of *A. thaliana* (Debeaujon, Peeters, Léeon-Kloosterziel, & Koorneef, 2001), SlMtp77 of *S. lycopersicum* (Mathews et al., 2003), VvAm1 and VvAm3 of *V. vinifera* (Gomez et al., 2009), MtMate2 of *M. truncatula* (J. Zhao et al., 2011), BnTt12-1 of *B. napus* (Chai et al., 2009), MdMate1 and MdMate2 of *M.* x *domestica* (Frank et al., 2011), VvMate1 and VvMate2 of *V. vinifera* (Pérez-Díaz et al., 2014), and GaTt12a of *G. arboretum* (Fuzhen, Xinmian, & Chengfu, 2012). Additionally, two ABC transporters shown to transport anthocyanins were included: ZmMrp3 of *Z. mays* (Goodman et al., 2004) and VvAbcc1 of *V. vinifera* (Francisco et al., 2013). Of these, AtTt12, VvAm1, VvAm3, and VvAbcc1 had already been functionally expressed in yeast microsomal fractions (Francisco et al., 2013; Gomez et al., 2009; Marinova et al., 2007) and AtTt12 had been shown to transport cyanidin-3-O-glucoside (Marinova et al., 2007). Plasmids expressing the coding sequences of the transporters and an empty plasmid as negative control were co-transformed, with two additional plasmids expressing *FaA3GT2* and *VvGST4*, into strain CYA2. After 72 hours of growth, yeast cells were separated from the culture broth by centrifugation. Supernatants and pellets were extracted and quantified separately, in order to see the distribution of cyanidin-3-O-glucoside (Figure 21). None

of the transporters resulted in increased production of cyanidin-3-O-glucoside or in increased transport of cyanidin-3-O-glucoside to the broth.

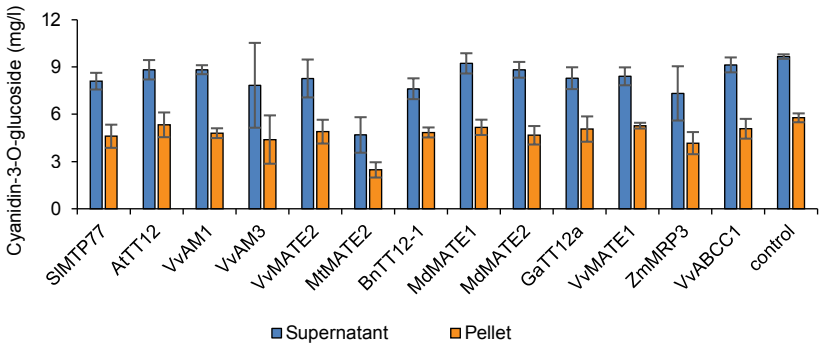


**Figure 21.** Testing anthocyanin transporters in the pathway to cyanidin-3-O-glucoside. Accumulation of cyanidin-3-O-glucoside in supernatants and pellets of strains CTR1-CTR14, co-expressing different transporters with *VvGST4* and *FaA3GT2* in strain CYA2. Represented are average and standard deviation of at least three independent cultures.

### 5.3.3 Production of anthocyanins in slow glucose release medium

Generally, glucose limited fed-batch fermentations are used for industrial production processes with Crabtree positive yeast strains like *S. cerevisiae*. This reduces the amount of aerobic fermentation, resulting in a lower production of by-products like ethanol or acetate and an increased biomass yield (Porro, Sauer, Branduardi, & Mattanovich, 2005). Expression of enzymes can vary significantly between batch and fed-batch cultivations (Scheidle et al., 2010). Therefore, screening and characterization of strains in small-scale cultures should also be performed in conditions with slow glucose release. Strains PGST1, CGST1, and DGST1, co-expressing the GST *PhAN9* together with the full-length pathways to pelargonidin-3-O-glucoside, cyanidin-3-O-glucoside, and delphinidin-3-O-glucoside, respectively, were selected in order to evaluate the potential for production of anthocyanins. Therefore, they were grown in 96 DWPs in a minimal medium buffered at pH6.4 with slow enzymatic glucose release, using the Feed in Time™ technology (Hemmerich, Wenk, Lütkepohl, & Kensy, 2011). Production of anthocyanins, intermediates, and by-products was followed for 120 hours (Figure 22). This strategy resulted in *de novo* biosynthesis of up to 179 ± 5 mg/l of pelargonidin-3-O-glucoside, 176 ± 4 mg/l of cyanidin-3-O-glucoside, and 65.8 ± 2.2 mg/l of delphinidin-3-O-glucoside. Phloretic acid, phloretin, and *p*-coumaric acid were the major by-products and intermediates observed. While compounds generally

accumulated over time, *p*-coumaric acid was consumed towards the end of the experiment after an initial buildup over the first 2-3 days.

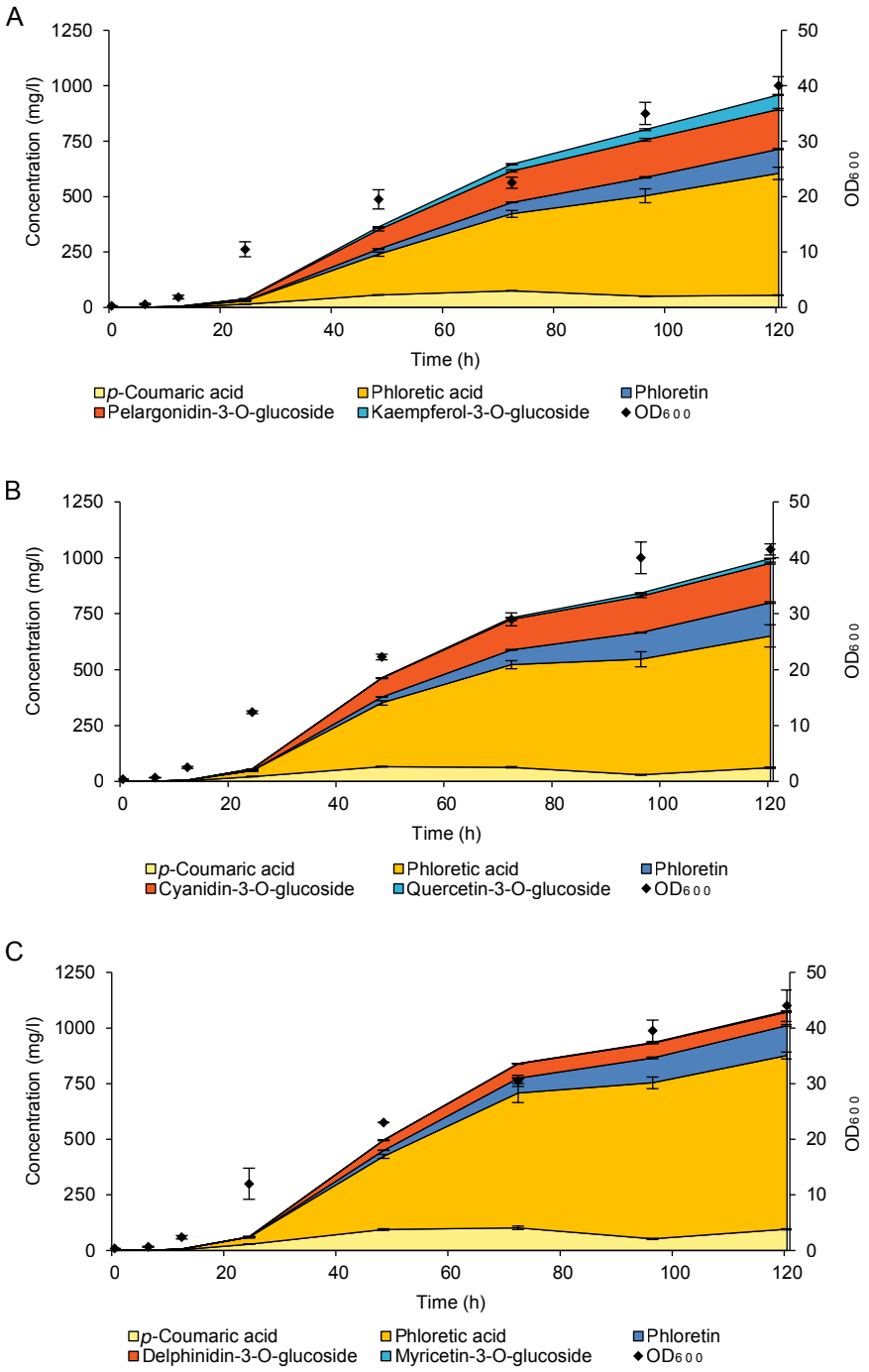


**Figure 22.** Production of anthocyanins, intermediates and byproducts by strains PGST1 (A), CGST1 (B), and DGST1 (C) over 120 hours growth in 96 DWP in slow glucose release medium buffered at pH 6.4. Represented are average and standard deviation of at least three independent cultures.

### 5.3.4 Modification of core anthocyanins results in various color formation

Commercially interesting anthocyanins are generally decorated with multiple sugar units, which are in many cases further acylated. This leads to increased stability of the molecules and determines their color properties (Wrolstad & Culver, 2012). Therefore, we aimed at showing *de novo* production of several decorated anthocyanins. To this end, several enzymes previously shown to be involved in such reactions were selected: Dv3mat catalyzes the malonylation of the 6''- hydroxygroup of the 3-O-glucose moiety of anthocyanins (Suzuki et al., 2002), VaA5gt glycosylates anthocyanidin-3-O-glucosides on the 5-hydroxygroup (He et al., 2015), CtA3'5'gt glycosylates the 3' and 5' hydroxygroups of cyanidin and delphinidin type anthocyanins (Noda et al., 2017), and NsA3grhat transfers a rhamnosyl group to the 6''-OH position of the 3-O-glucose moiety of anthocyanins to form anthocyanin rutinosides (Frydman et al., 2013). Strains PEL2, CYA2, and DEL2 were co-transformed with a plasmid expressing *PhAN9*, an empty *LEU2* plasmid, and all DNA fragments required for *in vivo* assembly of HRT plasmids comprising the genes of various combinations of decorating enzymes (Table 11). After 72 hours of growth in SC minimal medium, anthocyanin production was analyzed. Peaks with the exact mass of the expected final products, as listed in Table 11, were detected for all 18 strains. Except for the anthocyanidin-3-O-glucosides and the anthocyanidin-3,5-O-diglucosides, no authentic standards were commercially available and they could therefore not be quantified. Cyanidin-3,3'-O-diglucoside and delphinidin-3,3'-O-diglucoside co-eluted with cyanidin-3,5-O-diglucoside and delphinidin-3,5-O-diglucoside, respectively. Therefore, they were both quantified with authentic standards of the 3,5-O-diglusocides. Figure 23 shows the quantification of anthocyanin mono- and diglucosides. In most cases, upon expression of decorating enzymes the concentration of the precursors was decreased. To visualize the color properties of the produced anthocyanins, all 18 strains were grown in Feed in Time™ medium buffered to pH6.4 or pH4.0 in 24 DWPs for 72 hours (Figure 24). While decoration mainly changed the intensity of the colors in the low pH medium, it resulted in various new colors in the high pH medium, ranging from purple over green to blue tones.

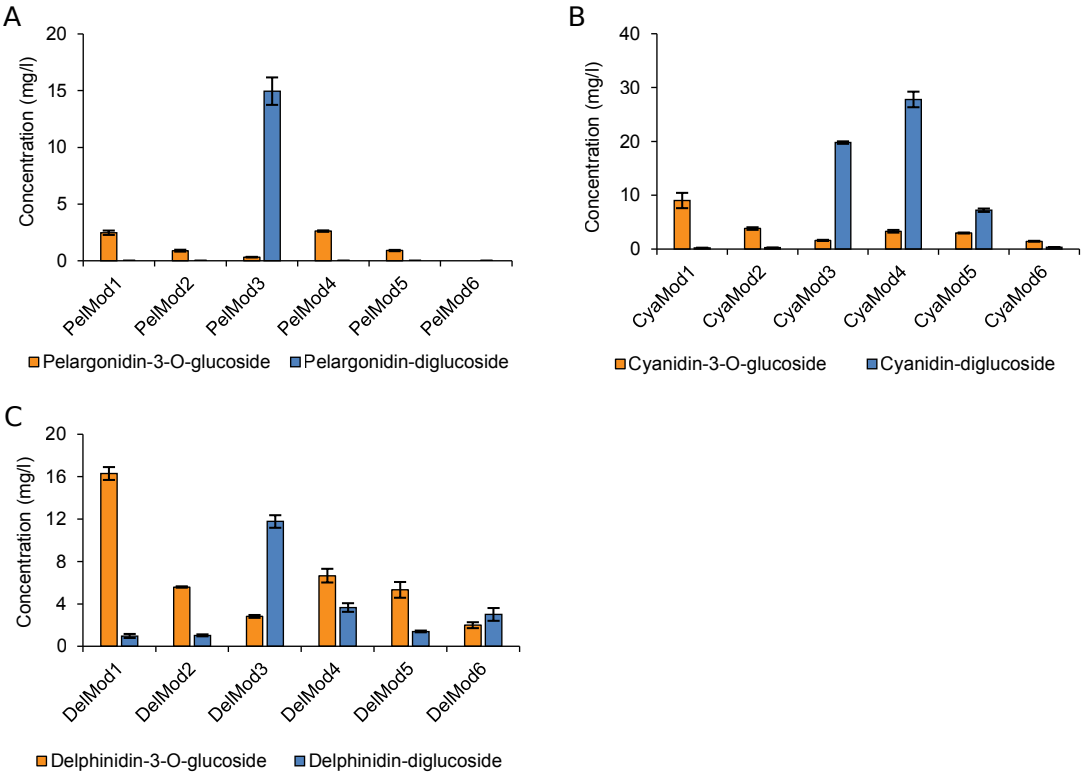


**Figure 23.** Testing further decoration of anthocyanins. (A) Production of pelargonidin-3-O-glucoside and pelargonidin-3-5-O-diglucoside of strains PMOD1-PMOD6, co-expressing various decorating enzymes, as shown in Table 11, and *PhAN9* in strain PEL2. (B) Production of cyanidin-3-O-glucoside and cyanidin-diglucosides of strains CMOD1-CMOD6, co-expressing various decorating enzymes, as shown in Table 11, and *PhAN9* in strain CYA2. (C) Production of delphinidin-3-O-glucoside and delphinidin-diglucosides of strains DMOD1-DMOD6, co-expressing various decorating enzymes, as shown in Table 11, and *PhAN9* in strain DEL2. Represented are average and standard deviation of at least three independent cultures.

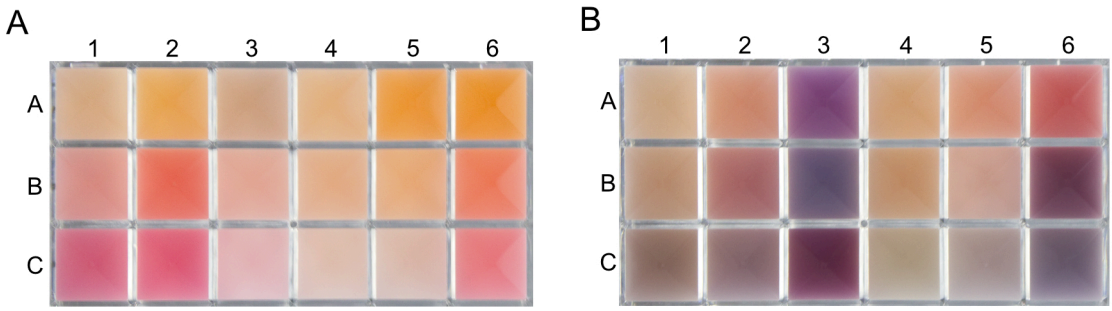


**Figure 24.** Colors of broth from strains producing various decorated anthocyanins after 72 hours of growth of strains PMOD1-PMOD6 (A1-A6), CMOD1-CMOD6 (B1-B6), and DMOD1-DMOD6 (C1-C6) in slow enzymatic glucose release, buffered to pH4.0 (A) and pH6.4 (B).

**Table 11.** Expected compounds after co-transformation of HRT reactions Mod1-Mod6 with a plasmid expressing *PhAN9* in strains PEL2, CYA2, and DEL2. In brackets are the coordinates corresponding to the 24 DWP in Figure 24

| HRT | Decorating genes | Expected compound in strain PEL2 | Expected compound in strain CYA2 | Expected compound in strain DEL2 |
|---|---|---|---|---|
| Mod1 | *FaA3GT2* | Pelargonidin-3-O-glucoside (A1) | Cyanidin-3-O-glucoside (B1) | Delphinidin-3-O-glucoside (C1) |
| Mod2 | *FaA3GT2 Dv3MAT* | Pelargonidin-3-O-(6''-O-malonyl) glucoside (A2) | Cyanidin-3-O-(6''-O-malonyl) glucoside (B2) | Delphinidin-3-O-(6''-O-malonyl) glucoside (C2) |
| Mod3 | *FaA3GT2 VaA5GT* | Pelargonidin-3,5-O-diglucoside (A3) | Cyanidin-3,5-O-diglucoside (B3) | Delphinidin-3,5-O-diglucoside (C3) |
| Mod4 | *FaA3GT2 CtA3'5'GT* | Pelargonidin-3-O-glucoside (A4) | Cyanidin-3,3'-O-diglucoside (B4) | Delphinidin-3,3',5'-O-triglucoside (C4) |
| Mod5 | *FaA3GT2 CtA3'5'GT Dv3MAT* | Pelargonidin-3-O-(6''-O-malonyl) glucoside (A5) | Cyanidin-3-O-(6''-O-malonyl) glucoside-3'-O-glucoside (B5) | Delphinidin-3-O-(6''-O-malonyl) glucoside-3',5'-O-diglucoside (C5) |
| Mod6 | *FaA3GT2 NsA3GRHAT AtRHM2* | Pelargonidin-3-O-rutinoside (A6) | Cyanidin-3-O-rutinoside (B6) | Delphinidin-3-O-rutinoside (C6) |

### 5.3.5 Discussion

The last step in the biosynthesis of the anthocyanin backbone is catalyzed by the enzyme anthocyanidin synthase (ANS), also called leucoanthocyanidin dioxygenase (LDOX). The first cDNA clone encoding an ANS enzyme was isolated from maize over 30 years ago (Menssen et al., 1990) and the first biochemical evidence for its involvement in conversion of leucoanthocyanidins to anthocyanidins was presented almost twenty years ago (Saito, Kobayashi, Gong, Tanaka, & Yamazaki, 1999). However, *in vitro* (Turnbull et al., 2000, 2003), in *E. coli* (Yan et al., 2008), and in *S. cerevisiae* (see 5.2.4), the enzyme converted leucoanthocyanidins mainly into dihydroflavonols and flavonols. Here we show that co-expression of plant GSTs, reported to be involved in anthocyanin transport, with the full anthocyanin pathway in yeast results in increased anthocyanin production and a reduction of flavonol by-product formation.

Several plant mutants of these GSTs have been isolated: *bronze2* in *Z. mays* (Marrs et al., 1995), *an9* in *P.* x *hybrida* (Alfenito et al., 1998), *fl3* in *D. caryophyllus* L. (Larsen et al., 2003), and *tt19* in *A. thaliana* (Kitamura et al., 2004). They all result in mislocalization and decreased content of anthocyanins. Initially, the GSTs were thought to transfer glutathione to anthocyanins, in order to label them for transport into the vacuole (Alfenito et al., 1998; Marrs et al., 1995). Several lines of evidence later proved

this hypothesis wrong: i) glutathione conjugates of anthocyanins could not be detected by *in vitro* reactions with GST or by extraction from plant material (Mueller et al., 2000), ii) a *bronze2* (S9A) variant devoid of glutathione transferase activity was still able to complement the *bronze2* phenotype (Mueller & Walbot, 2001), and iii) depletion of cellular glutathione or inhibition of GST activity in *A. thaliana* did not result in decreased anthocyanin accumulation (Poustka et al., 2007). On the other hand, both PhAn9 and AtTt19 were shown to bind anthocyanidins and anthocyanins. This led to the new hypothesis of GSTs being carrier proteins required for vacuolar transport. Their function might be to shuttle anthocyanins between the biosynthetic enzymes and transporters, to prevent oxidation of the instable anthocyanidins, or to prevent the cells from cytotoxic and genotoxic effects (Chanoca et al., 2015; Y. Huang et al., 2012; Mueller et al., 2000).

We propose an alternative model, in which the low level accumulation of anthocyanins in plant GST mutants is due to ANS mainly converting leucoanthocyanidins into flavonols instead of anthocyanidins in the absence of GST. This model is supported by several observations previously made in plants. First, the *A. thaliana* allele *tt19-7*, containing a GST with premature stop codon, resulted in seedlings accumulating only 11% of anthocyanins compared to wild type plants, while at the same time flavonol content increased by 36% (Sun et al., 2012). While this was explained by transcriptional upregulation of FLS, it might also be caused by increased flavonol production by ANS in the absence of GST. A second observation is linked to proanthocyanidin biosynthesis in *A. thaliana*. In this plant, proanthocyanidins are derived only from epicatechin monomers, which are polymerized in the vacuole. Epicatechin is synthesized from anthocyanidins by the action of anthocyanidin reductase in the cytoplasm (Figure 5) and transported into the vacuole by the MATE transporter AtTt12, most likely after 3'-O-glycosylation (J. Zhao & Dixon, 2009). Immature seeds from several *A. thaliana* mutants were compared for accumulation of soluble proanthocyanidins. While *tt12* mutants accumulated low but significant levels of epicatechin monomers and short chain oligomers, these compounds were completely absent in GST mutants *tt19* (Kitamura et al., 2010). This suggests that AtTt19 has an additional function besides transport. This could be the suggested involvement in biosynthesis of anthocyanidins, an intermediate of epicatechin biosynthesis. Third is an observation that was made several times in mutants of *bronze2* in *Z. mays*, *an9* in *P.* x *hybrida*, and *fl3* in *D. caryophyllus* L: After complementation by particle bombardment

with GST expressing DNA constructs, a halo of cells with faint anthocyanin pigmentation is formed around the fully complemented cells (Alfenito et al., 1998; Conn et al., 2008; Larsen et al., 2003). If the GSTs were only involved in vacuolar transport of anthocyanins, the effect of complementation should be limited to the cells expressing the GSTs. Therefore, this further suggests that the GSTs are involved in the biosynthesis of anthocyanidins, which are then diffusing or being transported from complemented to non-complemented cells, resulting in this halo formation.

We speculate that GSTs interacts directly with ANS, thereby changing the mechanism of product formation by the ANS enzyme. However, further studies are required to understand the exact mechanisms. Experiments like pull-down assays, yeast two hybrid assays, FRET, *in vitro* reconstitution of the protein complex, or co-crystallization might shed more light onto any such potential interaction. Furthermore, a range of single, double, and triple mutants in plants or plant cell cultures of FLS, ANS, and GST should be able to address whether this effect of GSTs is also relevant in plants.

In order to further understand the interplay of GSTs and transporters, a range of plant transporters involved in anthocyanin transport was co-expressed with the full anthocyanin pathway in yeast. This did not result in any detectable changes in the distribution between intracellular and extracellular anthocyanins or any increase of production. Even without transporter, a large fraction of anthocyanins was found outside yeast. In fact, this is observed with most polyphenols produced in yeast, and is probably due to unspecific yeast transporters. In order to better characterize the plant transporters in the orthogonal yeast system, the endogenous transport would have to be disrupted first. Then, functional expression and incorporation into the plasma membrane of the transporters would need to be assessed, for example by using GFP-fusions. This may also require a change of signal peptide sequences, in order to redirect the transporter from tonoplast to the yeast plasma membrane. Such experiments might generate a better understanding of the interplay between GSTs and transporters and their involvement in vacuolar accumulation of anthocyanins in plants.

The potential of *S. cerevisiae* as a sustainable production platform of anthocyanins is shown in this work. In slow glucose release medium, titers reached 179 ± 5 mg/l of pelargonidin-3-O-glucoside, 176 ± 4 mg/l of cyanidin-3-O-glucoside, and 65.8 ± 2.2 of delphinidin-3-O-glucoside. This is more than an order of magnitude higher than previously reported titers in a polyculture of four engineered *E. coli* strains (Jones et al., 2017). Notably, the yeast strains used were non-optimized laboratory strains, with no

steps taken to reduce by-product formation or to improve the pool of precursor molecules. Phloretic acid and phloretin, which constitute the two major by-products when grown in fed-batch medium, arise from the action of the endogenous yeast enoyl-CoA-reductase ScTsc13, which is an essential enzyme involved in very long chain fatty acid elongation. Replacement of this enzyme with plant homologs would allow complete elimination of these two by-products (Lehka et al., 2017). Interventions resulting in further increase of flux into the aromatic amino acid and polyketide pathways are well established. Two recent studies on metabolic engineering for production of *p*-coumaric acid (Rodriguez et al., 2015) and resveratrol (M. Li et al., 2015) in *S. cerevisiae* demonstrated that increasing the pool of aromatic amino acids and cytoplasmic malonyl-CoA, increasing the copy number of rate limiting pathway enzymes, and performing a controlled fed batch fermentation can result in a substantial increase of product titers, reaching 1.93 g/l for *p*-coumaric acid and 531.41 mg/l for resveratrol.

Commercially relevant anthocyanins are generally further decorated by glycosylation and acylation. To demonstrate the versatility of our yeast platform, a range of glycosyltransferases and a malonyltransferase were co-expressed with the core anthocyanin pathways. This resulted in the expected enzymatic 5-, 3'-, and 5'-O-glycosylation, as well as malonylation and rhamnosylation of the 3-O-glucose moiety in yeast. Culture broths of these yeast strains displayed a variety of colors, ranging from red over blue and green to purple.

It suggests the feasibility of creating engineered yeast cell factories, capable of producing commercially relevant anthocyanins, with pre-selected combinations of modifications resulting in desired properties regarding color and stability.

# 6 Abbreviations

| | |
|---|---|
| ABC | ATP binding cassette |
| ANR | Anthocyanidin reductase |
| ANS | Anthocyanidin synthase |
| A3GT | Anthocyanidin-3-O-glycosyl transferase |
| BC | Before Christ |
| CHI | Chalcone isomerase |
| CHS | Chalcone synthase |
| CH3H | Chalcone 3-hydroxylase |
| CPR | Cytochrome P450 reductase |
| CYP | Cytochrome P450 |
| C4H | Cinnamate 4-hydroxylase |
| DBR | Double bond reductase |
| DFR | Dihydroflavonol-4-reductase |
| DNA | deoxyribonucleic acid |
| DWP | Deepwell microplate |
| EMA | European Medicines Agency |
| FDA | Food and Drug Administration |
| F3H | Flavanone 3β-hydroxylase |
| F3’H | Flavonoid 3’-hydroxylase |
| F3’5’H | Flavonoid-3’,5’-hydroxylase |
| FLS | Flavonol synthase |
| GST | glutathione-S-transferase |
| LAR | Leucoanthocyanidin reductase |
| MATE | Multidrug and toxin extrusion |
| HRT | Homologous recombination tag |
| $OD_{600}$ | Optical density at 600 nm |
| OMT | O-methyltransferase |
| PAL | Phenylalanine ammonia lyase |
| PCR | Polymerase chain reaction |
| STS | Stilbene synthase |
| UGT | UDP-dependent-glycosyltransferase |
| UPLC-MS | Ultra performance liquid chromatography - mass spectrometry |

| | |
|---|---|
| VLC | Very long chain |
| 2ODD | 2-Oxoglutarate-dependent dioxygenase |
| 4CL | 4-Coumarate-CoA ligase |
| % (v/v) | Percent volume/volume |

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# 8 Publications

Eichenberger M, Lehka BJ, Folly C, Fischer D, Martens S, Simón E, Naesby M. Metabolic engineering of *Saccharomyces cerevisiae* for *de novo* production of dihydrochalcones with known antioxidant, antidiabetic, and sweet tasting properties. *Metab Eng*, 39:80-89, January 2017.

Lehka BJ, Eichenberger M, Bjørn-Yoshimoto WE, Vanegas KG, Buijs N, Jensen NB, Dyekjær JD, Jenssen H, Simon E, Naesby M. Improving heterologous production of phenylpropanoids in *Saccharomyces cerevisiae* by tackling an unwanted side reaction of Tsc13, an endogenous double-bond reductase. *FEMS Yeast Res*, 17(1):fox004, January 2017.

Skjoedt ML, Snoek T, Kildegaard KR, Arsovska D, Eichenberger M, Goedecke TJ, Rajkumar AS, Zhang J, Kristensen M, Lehka BJ, Siedler S, Borodina I, Jensen MK, Keasling JD. Engineering prokaryotic transcriptional activators as metabolite biosensors in yeast. *Nat Chem Biol*,12(11):951-958, November 2016.

# 9 Patent applications

Naesby M, Zokouri Z, Fischer D, Eichenberger M, Hansson A. Production of anthocyanin from simple sugars. WO2017050853. Filed 24.09.2015. Patent Pending.

Naesby M, Simon E, Eichenberger M, Lehka BJ, Bjørn -Yoshimoto WE, Jensen NB, Dyekjær JD. Biosynthesis of phenylpropanoid and dihydrophenylpropanoid derivatives. WO2016193504. Filed 05.06.2015. Patent Pending.

# 10 Poster presentations

Plant polyketides beyond Resveratrol – what yeast can do. SB 7.0, 2017, Singapore.

Metabolic engineering of *Saccharomyces cerevisiae* for *de novo* production of an antioxidant, an antidiabetic, and a sweet dihydrochalcone. XXVIIIth International Conference on Polyphenols, 2016, Vienna.
Poster Prize: Biosynthesis, Genetics & Metabolic Engineering

# 11 Curriculum vitae


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| 01/2015 - 12/2017 | **Evolva.** PhD Student. |
| 10/2013 - 12/2014 | **Evolva.** Research Associate. |
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| 06/2012 - 08/2012 | **ETH Zürich.** Scientist. |
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**Education**

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| 01/2015 - present | **TU Darmstadt.** Dr. rer. nat. in Biology. |
| | Doctoral thesis: *Production of polyketides in yeast.* |
| 09/2009 - 09/2012 | **ETH Zürich**. MSc in Biotechnology. |
| | Master thesis: *Immobilization of proteins in alginate beads.* |
| 10/2008 - 06/2009 | **Imperial College London**. Erasmus exchange. |
| | Bachelor thesis: *Novel homologous recombination based cloning techniques for engineering of modular, minimal plasmids.* |
| 10/2006 - 09/2009 | **ETH Zürich**. BSc in Biology (chemical branch of studies). |
| 08/2000 - 06/2006 | **Kantonsschule Zug**. Matura. |

# 12 Ehrenwörtliche Erklärung

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit entsprechend den Regeln guter wissenschaftlicher Praxis selbstständig und ohne unzulässige Hilfe Dritter angefertigt habe.

Sämtliche aus fremden Quellen direkt oder indirekt übernommenen Gedanken sowie sämtliche von Anderen direkt oder indirekt übernommenen Daten, Techniken und Materialien sind als solche kenntlich gemacht. Die Arbeit wurde bisher bei keiner anderen Hochschule zu Prüfungszwecken eingereicht.

Basel, den 10.02.2018

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